

## LOCATION OF RIBOSOMAL DNA IN CHROMOSOMES OF SQUAMATE REPTILES: SYSTEMATIC AND EVOLUTIONARY IMPLICATIONS

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**ABSTRACT:** In situ hybridization with biotinylated probes was used to determine the location of ribosomal DNA sequences in chromosomes of 16 species of lizards and four species of snakes. Variation was found among genera that are karyotypically identical according to non-differentially stained preparations. We conclude that during the evolution of squamate reptiles, the organization of rDNA sequences in the genome has changed substantially without altering the morphology of the karyotype. Closely related species and genera often have similar rDNA distribution patterns. For some groups, the location of rDNA may be of systematic value.

**Key words:** Ribosomal DNA; In situ hybridization; Genome organization; Chromosome evolution; Genetics; Squamata

GENES coding for ribosomal RNA in the nuclei of eukaryotes occur in tandemly-repeated sequences usually of several hundred copies (Gerbi, 1985). In mammals, these rDNA sequences may occur at a single location in the genome, or may be distributed on several chromosomes (Henderson et al., 1977; Hsu et al., 1975). Studies of the location of rDNA can give insight into patterns of genome evolution and location of rDNA sequences in monophyletic groups. Among some groups of reptiles, variation in the location of rDNA sequences has been found mainly between higher taxa, rather than among closely related species (Bickham and Rogers, 1985; King et al., 1986).

Some studies (Bickham and Rogers, 1985; Mahony and Robinson, 1986; Moritz, 1986; Peccinini-Seale and Alameida,

1986; Sites, 1983; Ward and Cole, 1986) have used silver-staining techniques to determine the location of nucleolar organizing regions (NOR's) in amphibians and reptiles. These NOR's frequently occur as chromosomal satellites delimited by a secondary constriction. The silver-staining technique is useful in determining the location of rDNA sequences that are being actively transcribed. However, in some cases (Ward and Cole, 1986), the rDNA sequences may be inactivated or lost, thus preventing the visualization of a NOR by silver-staining methods.

The procedure of in situ hybridization makes possible the location in the karyotype of specific repeated DNA sequences (King et al., 1986; Moritz, 1986; Pardue and Gall, 1970; Vitelli et al., 1982). In this technique, a single-stranded probe DNA molecule is bound to a denatured chromosome in those regions that have partially complementary base pairs. In our

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study, the probe molecule was a portion of the 28S ribosomal sequence of the laboratory mouse. The probe is biotin-labeled and treated so that the regions of hybridization are visible by fluorescent light microscopy.

The purpose of this study was to use in situ hybridization with the mouse 28S ribosomal probe to locate the repeated rDNA gene sequences in the genomes of various squamate reptiles, and to determine the pattern of rDNA sequence distribution with respect to the systematic relationships of the species examined.

#### MATERIALS AND METHODS

Specimens were collected from natural populations, and chromosome preparations were made from testes or bone marrow (Porter and Sites, 1986) in lizards, or from blood (Baker et al., 1971) in snakes. Representatives of five families of lizards (following the iguanian taxonomy of Frost and Etheridge, 1989) and two families of snakes were examined. Collecting localities are listed in Appendix I. Voucher specimens are deposited in research collections at The Museum, Texas Tech University (TTU), the M. L. Bean Life Science Museum, Brigham Young University (BYU), or the Instituto de Biología, Herpetología (IBHED) at the Universidad Nacional Autónoma de México in Mexico City. Collection, care, and sacrifice of specimens were by humane methods, following established guidelines and regulations (Committee, 1987).

Chromosomes in diakinesis, metaphase II, or mitotic metaphase were used in molecular hybridization treatments. Individual macrochromosomes were identified by size and centromere position, and were numbered from largest to smallest. In iguanian species with the 12 + 24 or 12 + 22 karyotype (six pairs of macrochromosomes and 11 or 12 pairs of microchromosomes, see below), all macrochromosomes are biarmed, with pair 2 submetacentric and all other pairs metacentric. Pairs 3 and 4 are identical in size and morphology, but the remaining macrochromosomes (pairs 1, 2, 5, and 6) are easily distinguishable from pairs 3 and 4

and from each other. Chromosomes of snakes and teiids are identified and numbered according to published descriptions (see references in Table 1).

Chromosome slides were flame dried by igniting a cell suspension immediately after it was dropped on the slides. In situ hybridization was performed with the I-19 ribosomal probe isolated by Arnheim (1979) from the 28S rDNA sequence of the laboratory mouse, *Mus musculus*, according to his procedures (Arnheim, 1979). In situ hybridization techniques are essentially those of Moyzis et al. (1987) as modified by Baker and Wichman (1990) and Hamilton et al. (1990). Chromosome preparations were treated with RNase (100  $\mu\text{g}/\text{ml}$ ) for 1 h at 37 C, followed by rinsing in four changes of 2X SSC, and sequential dehydration in 70%, 80%, and 95% ethanol. Slides were denatured for 2 min at 70 C with 70% formamide (Kodak ACS) in 2X SSC, followed by immersion in 70% ethanol at -10 C. The slides were then dehydrated by sequential treatment in 80% and 95% ethanol. Hybridization was performed under sealed coverslips for 12-18 h at 37 C. The hybridization solution consisted of approximately 1-3  $\mu\text{g}$  biotinylated probe DNA (biotin-labeled with a Clontech Biotin-21-dUTP Nick Translation Kit), per ml of 2X SSC, 500  $\mu\text{g}/\text{ml}$  *Escherichia coli* carrier DNA, and 30% formamide. The probe was denatured by heating the hybridization mixture to 70 C for 5 min and then immediately cooling to 0 C. Following hybridization, the slides were washed for 2 min each in five changes of 2X SSC, pH 7.0 at 40-42 C. Hybridization was detected by binding fluorescein-labeled avidin (Vector Laboratories; 3  $\mu\text{l}$  avidin and 0.05 g bovine albumin in 1 ml BT buffer) to biotin-labeled probes for 1 h at 37 C. The preparations were amplified at least once with biotinylated goat anti-avidin antibody (Vector Laboratories; 7.5  $\mu\text{g}$  antibody per ml 5% goat serum) applied for 1 h at 37 C, followed by five washes of 2 min each in BT buffer at 40-42 C, and then by an additional avidin treatment. In some instances, two amplifications provided the best visualization. Slides were counterstained with propidium io-

TABLE 1.—Location of hybridization of the rDNA probe for each taxon studied. The number of acrocentric (A) and biarmed (B) macrochromosomes is indicated, along with the number of microchromosomes (m).

Taxon	A	B	m	Location of hybridization	Karyotype references*
<b>Crotaphytidae</b>					
<i>Crotaphytus collaris</i>	0	12	24	long arm of pair 2†	17
<b>Phrynosomatidae</b>					
<i>Cophosaurus texanus</i>	0	12	22	pair 3 or 4†	5, 21
<i>Holbrookia maculata</i>	0	12	22	pair 3 or 4†	1, 5‡
<i>Phrynosoma cornutum</i>	0	12	22	one pair microchromosomes	6
<i>Uta stansburiana</i>	0	12	22	one pair microchromosomes	16
<i>Sceloporus magister</i>	0	12	14	long arm of pair 2†	13, 18
<i>Sceloporus graciosus</i>	0	12	18	long arm of pair 2†	11, 14
<i>Sceloporus undulatus</i>	0	12	10	long arm of pair 2†	9
<i>Sceloporus grammicus</i> (Low S cytotype)§	0	12	20	long arm of pair 2†	3, 4, 7
<i>Sceloporus grammicus</i> (High S cytotype)§	0	12	20	long arm of pair 2†	3, 4, 7
<i>Sceloporus palaciosi</i> §	4	10	20	long arm of pair 2†	3, 4, 7
<b>Polychridae</b>					
<i>Anolis porcatus</i>	0	12	24	long arm of pair 1†	2, 15
<i>Anolis homolechis</i>	0	14	14	pair 2 or 3†	2, 15
<b>Tropiduridae</b>					
<i>Leiocephalus raviceps</i>	0	12	18	long arm pair 2† and one pair microchromosomes¶	2
<i>Leiocephalus carinatus</i>	0	12	22	long arm pair 2† and one pair microchromosomes¶	2
<b>Teiidae</b>					
<i>Ameiva auberi</i>	10	8	12	one pair microchromosomes	2
<i>Cnemidophorus marmoratus</i>	0	22	24	pair 2†	19
<b>Colubridae</b>					
<i>Masticophis flagellum</i>	2	14	20	one pair acrocentric microchromosomes#	8, 12
<i>Nerodia fasciata</i>	0	34	2	long arm pair 1 or 2†	10
<i>Thamnophis marcianus</i>	0**	34	2	long arm pair 1 or 2†	8
<b>Viperidae</b>					
<i>Crotalus viridis</i>	2	14	20	two pairs microchromosomes¶	8, 20

\* 1 = this study; 2 = Porter et al., 1989; 3 = Sites et al., 1987; 4 = Porter and Sites, 1986; 5 = Paull et al., 1976; 6 = Gorman, 1973; 7 = Hall, 1973; 8 = Baker et al., 1972; 9 = Cole, 1972; 10 = Eberle, 1972; 11 = Cole, 1971; 12 = Bury et al., 1970; 13 = Cole, 1970; 14 = Jackson and Hunsaker, 1970; 15 = Gorman and Atkins, 1968; 16 = Penneck et al., 1968; 17 = Cohen et al., 1967; 18 = Lowe et al., 1967; 19 = Lowe and Wright, 1966; 20 = Monroe, 1962; 21 = Painter, 1921.

† Hybridization restricted to telomeric end.

‡ Except for a remarkably accurate early description of the chromosomes of *C. texanus* (Painter, 1921), reviews of reptilian chromosomes (Gilboa, 1975; Gorman, 1973; Peccinini-Scale, 1981) do not list karyotypes for earless lizards. However, unpublished data of W. P. Hall (cited in Paull et al., 1976) indicate a diploid number of 34 for two unspecified species of *Holbrookia*. Our results for *H. maculata* were concordant with Hall's data.

§ These taxa are members of the *Sceloporus grammicus* complex. For information on the taxonomic status of these populations, see Arévalo et al. (1991), Hall (1973, 1980), Porter and Sites (1986), Sites (1983), Sites et al. (1988), and Sites and Davis (1989).

¶ 19 microchromosomes in males.

|| Hybridization restricted to one end of the microchromosomes.

# Hybridization restricted to the centromeric end.

\*\* One of the 34 macrochromosomes is acrocentric in females.

dide (Sigma) and 4,6-diamidino-2-phenylindole (DAPI; Sigma). Counterstaining with propidium iodide and viewing with ultraviolet light at 436 nm allows the simultaneous observation of the fluorescein-labeled hybridized probe (yellow) and total DNA (orange), while DAPI viewed by

a wavelength of 365 nm allows total DNA to be visualized. Counterstains were applied to slides in an antifade mounting medium (Johnson and Araujo, 1981) at concentrations of 0.25–0.4  $\mu\text{g}/\text{ml}$  (DAPI) and 1.5–2.0  $\mu\text{g}/\text{ml}$  (propidium iodide). Photographs were taken with Kodak Ektar

1000 film exposed at E.I. 2000 with an Olympus Epi-fluorescent microscope. In most cases, five or more cells were examined per individual.

#### RESULTS AND DISCUSSION

Table 1 lists the location of hybridization of the ribosomal probe and a summary of the karyotype for each taxon examined, and Fig. 1 illustrates representative cells.

Many species of iguanian lizards have a  $2N = 36$  karyotype with 12 biarmed macrochromosomes and 24 microchromosomes. As revealed by non-differential giemsa staining, this  $12 + 24$  karyotype is remarkably invariant in its macrochromosomal morphology in various families of lizards (Paull et al., 1976) and has been suggested as ancestral to iguanians (Bickham, 1984; Gorman et al., 1967; Paull et al., 1976). For example, families as divergent as Phrynosomatidae, Polycridae, and Chamaeleonidae have species that are virtually identical in macrochromosomal morphology. Although the  $12 + 24$  karyotype is common in a broad array of iguanian groups, many families also contain genera with extreme chromosomal variation. The  $12 + 24$  karyotype is found in all of the iguanian families recognized by Frost and Etheridge (1989), although a  $12 + 22$  karyotype is thought to be ancestral for the phrynosomatids (Paull et al., 1976). In spite of the apparent conservation of macrochromosomal morphology in the  $12 + 24$  karyotype, our data show that the species with this pattern do not always have identical patterns of rDNA distribution.

We discuss below the specific implications of rDNA distribution in each family studied.

##### *Family Crotophytidae*

*Crotaphytus collaris* retains the  $12 + 24$  karyotype thought to be ancestral for the iguanian lizards (Bickham, 1984; Gorman et al., 1967; Paull et al., 1976). The crotophytids were once thought to be the nearest relatives of the phrynosomatids (Etheridge in Paull et al., 1976), but more recently this hypothesized close relationship has been questioned (Etheridge and de Queiroz, 1988; Frost and Etheridge,

1989). The macrochromosomal karyotype is similar to that thought to be ancestral in the phrynosomatid genus *Sceloporus*, and the distribution of rDNA is indistinguishable in the species of *Crotaphytus* and *Sceloporus* studied.

##### *Family Phrynosomatidae*

A karyotype of  $2N = 34$  ( $12 + 22$ ) appears to be ancestral for this family (Bickham, 1984; Hall, 1973; Paull et al., 1976), and differs from the  $12 + 24$  karyotype only by the reduction of microchromosomes by one pair. Of the 8–10 recognized genera of this family, only the chromosomally variable genus *Sceloporus* differs from the presumed ancestral  $12 + 22$  karyotype (Paull et al., 1976). In many species of *Sceloporus*, the ancestral macrochromosomal pattern is retained, and most derived karyotypes differ in the number of microchromosomes or by centric fission of one or more macrochromosomes. Although they represent several different species groups within the genus (Smith and Taylor, 1950), the six taxa of *Sceloporus* that we studied differ mainly in the number of microchromosomes. The macrochromosomes in these species are identical in morphology, except in *S. palaciosi*, which is fixed for a centric fission of macrochromosome pair 6.

In *S. graciosus* and *S. magister*, the location of the ribosomal genes, as documented by in situ hybridization, is identical to the position of a previously identified distinct secondary constriction (Cole, 1971; Jackson and Hunsaker, 1970; Lowe et al., 1967). In both the high- and low-elevation *S.* ( $2N = 32$ ) cytotypes of *S. grammicus*, the ribosomal genes correspond to the location of a secondary constriction and silver-stained NOR's found in the F5+6 ( $2N = 36$ ) cytotype of the *S. grammicus* complex (Sites, 1983).

In the genus *Sceloporus*, hybridization was found at the telomeric end of the long arm of pair 2 (Fig. 1A). However, in *Phrynosoma cornutum* and *Uta stansburiana*, only a single pair of microchromosomes showed hybridization (Fig. 1B). In the earless lizards (*Holbrookia* and *Cophosaurus*), the signal was found on pair 3 or 4 (which

are distinguishable in non-differentially stained chromosomes; Fig. 1C). If derived, the similarity between *Cophosaurus* and *Holbrookia* supports the hypothesis of a close relationship between these genera (Etheridge and de Queiroz, 1988). Some authors (Norris, 1958; Paull et al., 1976) have considered the greater earless lizard (*Cophosaurus texanus*) to be more closely allied to the zebra-tailed lizard, *Callisaurus draconoides*, than it is to the other earless lizards (*Holbrookia*). Future studies of the location of rDNA in *Callisaurus* and *Uma* may help elucidate the relationships among these genera. If *Callisaurus* and *Uma* are found to have microchromosomal rDNA, then the rDNA distribution in *Cophosaurus* and *Holbrookia* would be a synapomorphy uniting the earless lizards, and separating them from their nearest relatives. If *Callisaurus* and *Uma* have rDNA genes on pair 3 or 4, then this pattern may be a trait derived in the sand lizards.

Although we cannot determine the identity of the specific microchromosomes involved, the ribosomal gene organization may be the same in *P. cornutum* and *U. stansburiana*. These two genera represent two distinct lineages within the family. *Uta* is considered (Etheridge and de Queiroz, 1988; Paull et al., 1976) part of the *Sceloporus* group (in which our studies show the ribosomal sequences on pair 2), whereas *Phrynosoma* is thought (Etheridge and de Queiroz, 1988; Frost and Etheridge, 1989) to be allied with the sand lizards (at least two genera of which have species with ribosomal genes on pair 3 or 4). If the currently accepted phylogeny (Etheridge and de Queiroz, 1988) is correct, it is possible that *Phrynosoma* and *Uta* retain the ancestral phrynosomatid condition (with rDNA sequences on microchromosomes), while *Sceloporus* and the earless lizards have alternative derived states (rDNA sequences translocated to macrochromosome pair 2 in *Sceloporus* and to pair 3 or 4 in the earless lizards). Alternatively, it is possible that both *Crotaphytus* and *Sceloporus* retain the ancestral iguanian condition, while *Phrynosoma* and *Uta* independently evolved microchromosomal

rDNA sequences. The rock lizards (genus *Petrosaurus*) are considered by some authors to be a sister taxon of the remaining phrynosomatids (Etheridge and de Queiroz, 1988; but see Frost and Etheridge, 1989, who question the traits used to establish this relationship). If *Petrosaurus* is primitive in the phrynosomatids, its rDNA distribution may help resolve whether the *Sceloporus* or the *Uta-Phrynosoma* condition is ancestral for the family. If the rDNA distribution of *Sceloporus* is ancestral for the phrynosomatids, then the microchromosomal location of the rDNA sequences evolved independently at least twice in the family.

It appears that the organization of rDNA genes may have remained relatively unchanged in *Sceloporus*, in spite of extensive speciation and karyotypic evolution, whereas other phrynosomatid genera have altered their rDNA sequence distribution with no visible change in karyotype morphology.

#### Family Polychridae

The  $2N = 36$  ( $12 + 24$ ) karyotype of *Anolis porcatus* is common among the non-beta *Anolis* (Gorman, 1973; Gorman and Atkins, 1968) and is considered ancestral for the polychrids (Gorman, 1973; Gorman et al., 1967; Guyer and Savage, 1986). The 12 metacentric macrochromosomes appear identical in morphology to those found in many species in related families of iguanian lizards with the  $12 + 24$  karyotype. However, unlike other lizards with this macrochromosomal configuration, the ribosomal probe hybridized to chromosome pair 1 (Fig. 1D). If pair 2 is the ancestral location for iguanian rDNA, then the *A. porcatus* pattern seems to be derived.

The  $14 + 14$  karyotype of *Anolis homolechis* is representative of the *sagrai* series of the genus, and it is apparently derived from the  $12 + 24$  karyotype thought to be ancestral to the polychrids (Gorman, 1973; Gorman et al., 1967; Guyer and Savage, 1986). In *A. homolechis*, the ribosomal probe hybridized to either pair 2 or 3, which are similar in size and morphology. The homologies of *A. homolechis* chromosomes with other polychrid karyotypes

- Co.: 25.8 km S, 8.1 km E Post. *Holbrookia maculata maculata*. TK24265 (female). Texas: Lubbock Co.: Lubbock. *Leiocephalus carinatus*. TK24174 (male). Cuba: Guantánamo Province: Guantánamo Naval Base. *Leiocephalus raviceps*. TK24170 (male). Cuba: Guantánamo Province: Guantánamo Naval Base. *Masticophis flagellum testaceus*. TK24164 (female). Texas: Garza Co.: 22.6 km S, 1.6 km E Post. *Nerodia fasciata pictiventris*. TK24258 (female). Florida: Collier Co.: South of Hwy 84, between Naples and Miles City. *Phrynosoma cornutum*. TK24224 (male). Texas: Lubbock Co.: 3.2 km E Lubbock Airport. *Sceloporus graciosus graciosus*. TK24221 (female). Utah: Washington Co.: Leeds Canyon, approximately 13.7 km W Leeds in Oak Grove campground. *Sceloporus grammicus* (high elevation S cytotype). TK24270, IBHED 07163; (male). Mexico: Tlaxcala: Volcan Malinchi, approximately 3200 m elev., on dirt road above Albergue Malintzi. *Sceloporus grammicus microlepidotus* (low elevation S cytotype). TK24273, BYU39797; (male). Mexico: Distrito Federal: Col. Lindavista, C. C. H. Vallejo Del. G. A. Madero. *Sceloporus magister uniformis*. TK24223 (male). Utah: Washington Co.: Leeds Canyon, approximately 5.6 km W Leeds. *Sceloporus palaciosi*. TK24271, IBHED 07165; (male). Mexico: Michoacan: Cerro Burro along gravel road at approximately 2900–3000 m elev., about 4.0 km from intersection with Hwy 41, S of San Gregorio and SW of Patzcuaro. TK24272, IBHED 07174; (male) Mexico: Jalisco: Sierra del Tigre, carretera Mazamitla—torre de microondas El Montoso (approximately 8.0 km from Mazamitla) 2450–2700 m. *Sceloporus undulatus elongatus*. TK24222 (male). Utah: Washington Co.: Leeds Canyon, approximately 7.3 km W Leeds. *Thamnophis marcianus marcianus*. TK24189 (female). Texas: Lubbock Co.: 4.8 km W, 4.8 km N Idalou. *Uta stansburiana stejnegeri*. TK24285 (female). New Mexico: Doña Ana Co.: 27.4 km N Las Cruces.

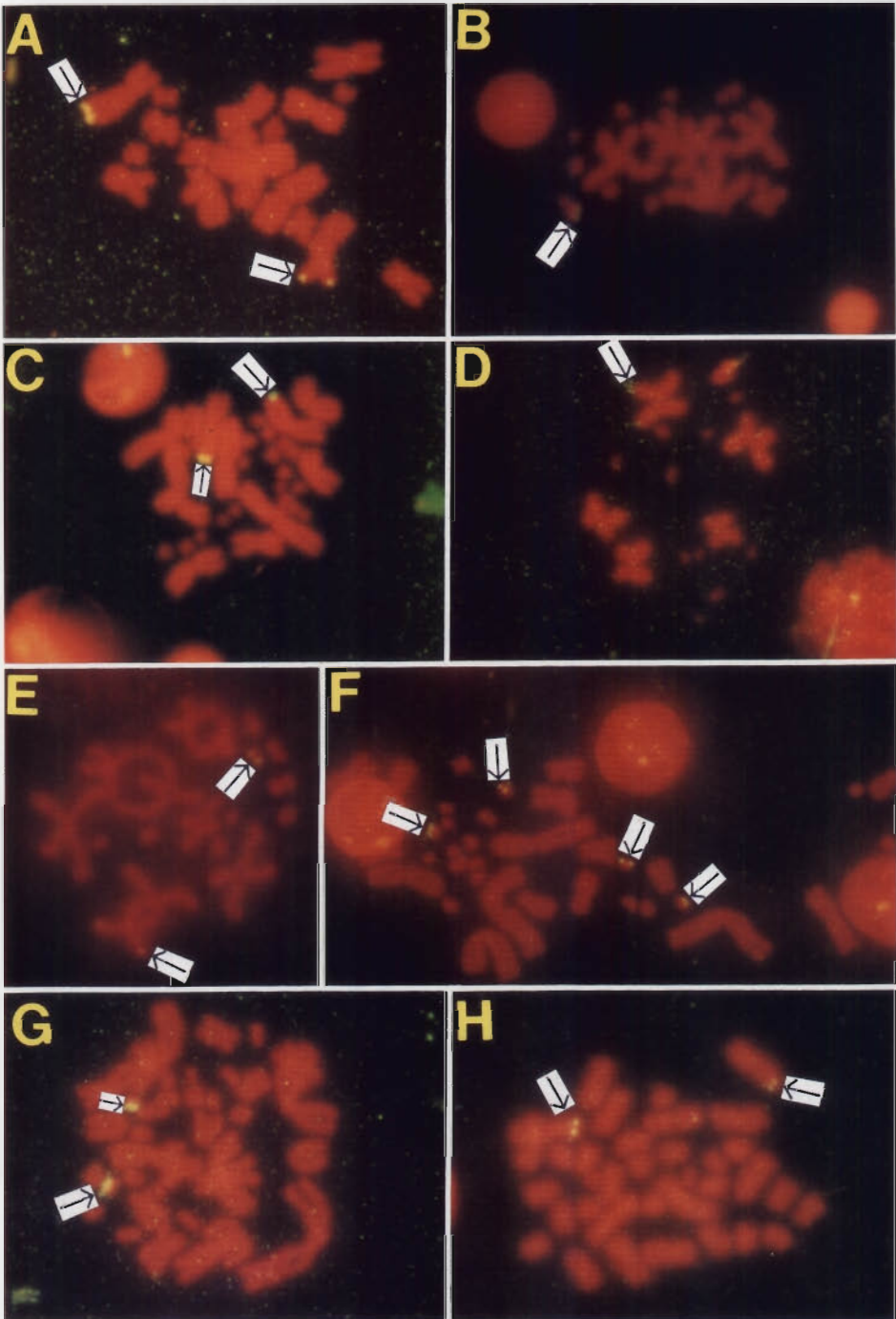


FIG. 1.—Hybridized karyotypes for representative species of snakes and lizards. Chromosomes that consistently showed hybridization (yellow regions) in all cells are indicated with an arrow. Other yellow spots not marked with an arrow are the result of background signal. (A) *Sceloporus undulatus*; (B) *Phrynosoma cornutum* haploid secondary spermatocyte; (C) *Holbrookia maculata*; (D) *Anolis porcatus* haploid secondary spermatocyte; (E) *Leiocephalus raviceps* diakinesis cell, showing hybridization to two bivalents; (F) *Crotalus viridis*; (G) *Masticophis flagellum*; (H) *Thamnophis marcianus*.