STEJNEGER, L. 1907. Herpetology of Japan and adjacent territory. Bull. U. S. Nat. Mus. 58:47–161.
STORM, R. M. 1947. Eggs and young of *Aneides ferreus*. Herpetologica 4:60–62.
TILLEY, S. G. 1972. Aspects of parental care and embryonic development in *Desmognathus ochrophaeus*. Copeia 1972:532–540.
VANDEL, A., AND BOUILLON, M. 1959. La réproduction du protée (*Proteus anguineus*), C. R. Acad. Sci. Paris 248:1267–1272.
VIAL, J. L. 1968. The ecology of the tropical salamander, *Bolitoglossa subpalmata*, in Costa Rica. Rev. Biol. Trop. 15:13–115.
———, AND F. B. PREIB. 1966. Antibiotic assay of dermal secretions from the salamander, *Plethodon cinereus* (Green). Herpetologica 22:284–287.
———, AND ———. 1967. An investigation of antibiosis as a function of brooding behavior in the salamander, *Plethodon cinereus*. Proc. Mo. Acad. Sci. 1:37–40.

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Ammoniacal Silver Staining of Nucleolar Organizer Regions in Four Species of *Bufo*

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The nucleolar organizer regions (NORs) of *Bufo americanus, B. woodhousei woodhousei, B. w. fowleri, B. valliceps* and *B. marinus* were studied using an ammoniacal silver procedure. In each taxon a NOR was observed in only one pair of chromosomes. The NOR occurred on chromosome 1 adjacent to the centromere in *B. americanus, B. w. woodhousei, B. w. fowleri* and *B. valliceps*. In *B. marinus* the NOR was located on chromosome 7. The Ag-NORs corresponded to secondary constrictions detectable as achromatic gaps in Giemsa-stained metaphase chromosomes.

THE chromosomes of most *Bufo* species show striking similarities with the major reported variation being in the position of secondary constrictions (Bogart, 1972). Bogart (1972) utilized these secondary constrictions to infer various dichotomies in the evolution of *Bufo* karyotypes. The number and position of secondary constrictions have been used as markers in karyotypic studies of numerous amphibians (Hennen, 1964; Seto, 1965; Robinson and Stephenson, 1967; Wasserman and Bogart, 1968; Bogart, 1972; Haertel et al., 1974). However, secondary constrictions have been shown to be subject to considerable variation depending on preparative techniques (Sasaki and Makino, 1963; Palmer and Funderburk, 1965; Callan, 1966; Bruere and McLaren, 1967).

Secondary constrictions are usually associated with nucleolar formation and are referred to as nucleolar organizer regions (NORs). In situ hybridization studies have shown that the cistrons coding for the 18s and 28s ribosomal RNA are located at the NORs (Pardue et al., 1970; Pardue, 1974; Henderson et al., 1972, 1974; Pardue and Hsu, 1975). However, Hsu et al. (1975) have demonstrated that not all secondary constrictions bind radioactive ribosomal RNA whereas some regions of metaphase chromosomes bind radioactive ribosomal RNA but do not appear as secondary constrictions. In addition, cold treatment of metaphase chromosomes induces the occurrence of secondary constrictions other than those corresponding to the NORs (Rudak and Callan, 1976). Hence, not all secondary constrictions correspond to nucleolar organizer regions.

Goodpasture and Bloom (1975) have recently developed an ammoniacal silver technique for demonstrating NORs in mammalian cells. This technique apparently stains the same chromo-

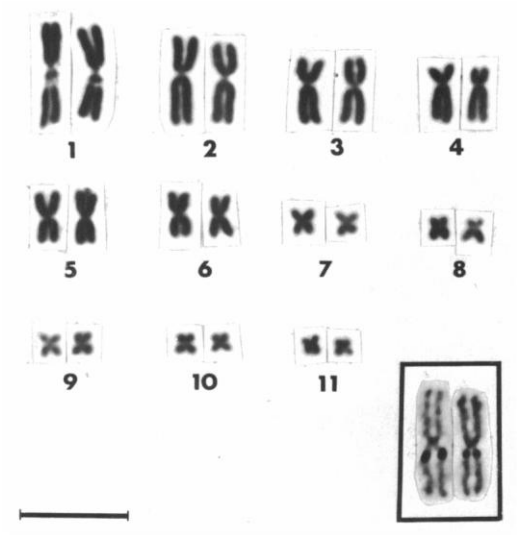

Fig. 1. Karyotype of *Bufo americanus* (female). Ag-NOR chromosome are shown in inset. Bar equals 10 microns.

somal regions demonstrable by in situ hybridization. Ward (1977) employed this silver technique for studying dimorphic NORs in the frog *Rana blairi*.

This study was undertaken to determine the number and position of NORs in the metaphase chromosomes of four species of *Bufo*.

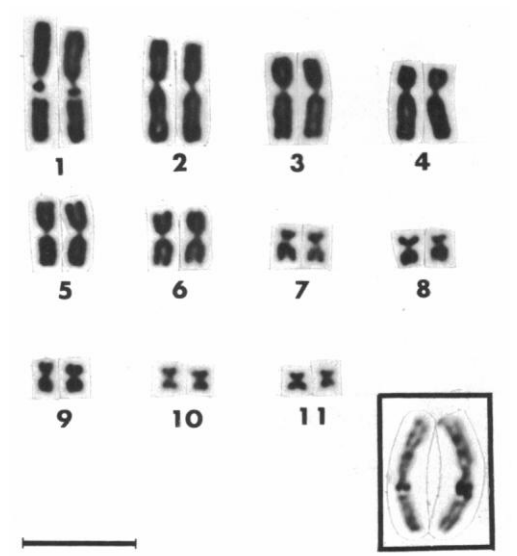

Fig. 2. Karyotype of *Bufo woodhousei woodhousei* (male). Ag-NOR chromosomes are shown in inset. Bar equals 10 microns.

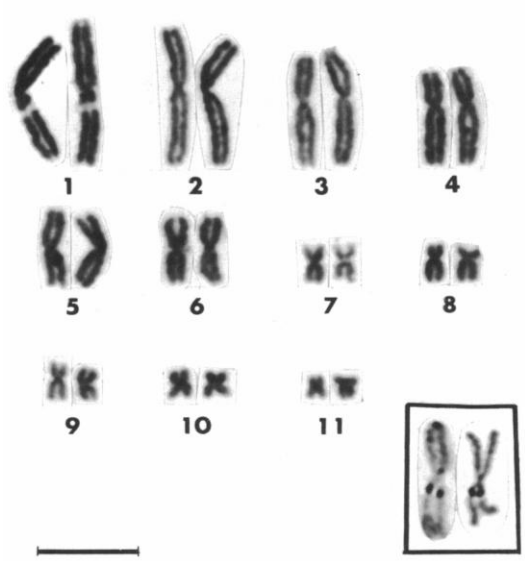

Fig. 3. Karyotype of *Bufo woodhousei fowleri* (male). Ag-NOR chromosomes are shown in inset. Bar equals 10 microns.

Materials and Methods

Animals.—The location of NORs in metaphase chromosomes of four species of *Bufo* was studied using the ammoniacal silver technique of Goodpasture and Bloom (1975). The animals studied were obtained from the following localities: 1) *B. americanus*: Oshkosh, Wisc. (2 ♂♂, 2 ♀♀); Only, Tenn. (1 ♀); Memphis, Tenn. (1 ♂, 2 ♀♀). 2) *B. woodhousei woodhousei*: Norman, Okla. (4 ♂♂, 3 ♀♀). 3) *B. woodhousei fowleri*: Horn Lake, Miss. (5 ♂♂, 1 ♀); Chickasaw, Tenn. (1 ♂, 2 ♀♀); Sugar Tree, Tenn. (1 ♂, 1 ♀). 4) *B. valliceps*: Groves, Texas (11 ♂♂, 3 ♀♀). 5) *B. marinus*: McAllister, Texas (1 ♀).

Cytological preparations.—Blood was obtained from the femoral artery and transferred to culture vials containing 5 ml of Wolf and Quimby amphibian culture medium (GIBCO), 0.2 ml of phytohemagglutin M, 0.1 ml of a penicillin-streptomycin mixture (DIFCO) and 0.1 ml of nystatin (50 mcg/ml). Cultures were incubated at 26 C for 5 days. Six hours prior to harvest, colchicine was added to each culture to give a final concentration of 2×10^{-5} M. The cells were treated with a hypotonic solution of 0.075 M potassium chloride or 1.0% sodium citrate for 15 min and fixed in methanol:acetic acid

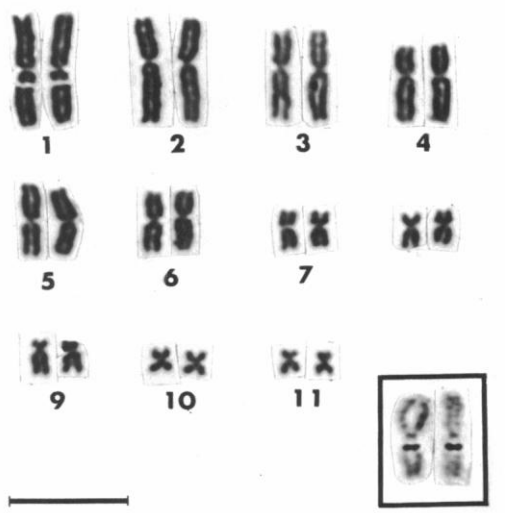

Fig. 4. Karyotype of *Bufo valliceps* (female). Ag-NOR chromosomes are shown in inset. Bar equals 10 microns.

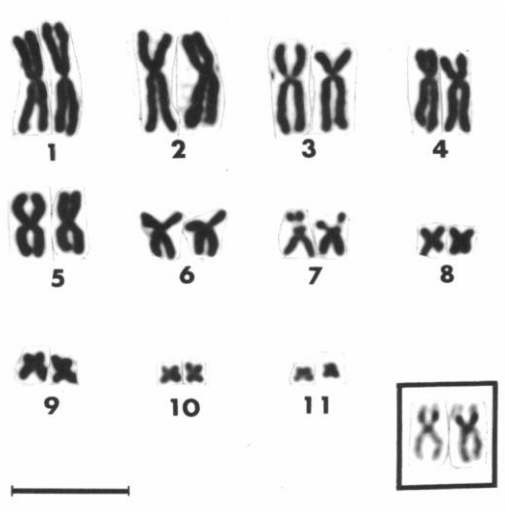

Fig. 5. Karyotype of *Bufo marinus* (female). Ag-NOR chromosomes are shown in inset. Bar equals 10 microns.

(3:1). Cells were dropped onto clean slides and air dried.

Staining procedure.—Chromosomal NORs were stained according to the Ag-As procedure of Goodpasture and Bloom (1975). Freshly prepared slides or ones that had been stored several months gave identical staining results. Some slides were stained with 2% Giemsa for preparation of standard karyotypes. In all karyotypes, the chromosomes were arranged in order of decreasing lengths.

Results

All the animals examined possessed a diploid number of 22 and the karyotypes were very similar (Figs. 1–5). In Giemsa-stained preparations, chromosome 1 of *B. americanus, B. w. woodhousei, B. w. fowleri* and *B. valliceps* exhibited a prominent achromatic secondary constriction adjacent to the centromere (Figs. 1–4). However, in *B. marinus* the secondary constriction was observed in the short arm of chromosome 7 instead of on chromosome 1 (Fig. 5). Secondary constrictions were not observed on any other chromosomes in Giemsa-stained preparations. The position of secondary constrictions was consistent in each species and appeared constant in a given chromosome.

After Ag-As staining, the NORs appeared as dark black regions on light brown chromosomes (Fig. 6). The Ag-NORs corresponded exactly to the achromatic secondary constrictions that had been observed in the Giemsa-stained preparations (Figs. 1–5). No more than two chromosomes exhibited Ag-NORs in any cell examined. Thus the NORs appeared to be confined to a single pair of chromosomes in each *Bufo* species examined.

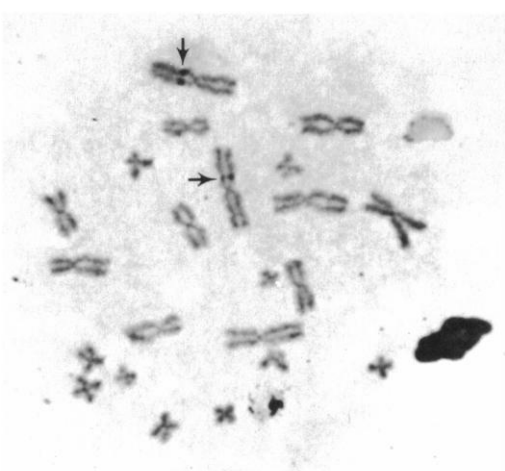

Fig. 6. *Bufo valliceps* (female) chromosomes stained by the Ag-As method. Note the distinct staining of the NORs (arrows). ×1500.

Even though the position of the Ag-NORs was constant in the karyotype of each species, it was not necessarily the same size on homologous chromosomes. The Ag-NORs of *B. w. woodhousei* were often observed to be of unequal size with one homolog possessing a larger NOR (Fig. 2).

Discussion

The number and position of secondary constrictions have been utilized to a considerable extent in comparative karyological studies of the genus *Bufo* (Moreschalchi and Garauilo, 1968; Bogart, 1972). Bogart (1972) identified three secondary constrictions from karyotypes of *B. americanus* and six from karyotypes of *B. valliceps* whereas *B. woodhousei* and *B. marinus* possessed a single secondary constriction on chromosomes 1 and 7 respectively. In this study *B. woodhousei* and *B. marinus* were found to possess only a single secondary constriction as reported previously by Cole et al. (1968) and Bogart (1972). In addition, only a single achromatic secondary constriction was found adjacent to the centromere of chromosome 1 in all karyotypes of *B. americanus* and *B. valliceps*. No other secondary constrictions were observed in metaphase chromosomes of these two species. Cole et al. (1968) also reported only a single distinct secondary constriction near the centromere of chromosome 1 in *B. valliceps*. The occurrence of secondary constrictions appears to be influenced by preparative techniques and also differs from tissue to tissue. Bruere and McLaren (1967) reported that the incidence of secondary constrictions was influenced by the type of hypotonic treatment. The colchicine concentration (Palmer and Funderburk, 1965) and calcium content of the culture medium (Sasaki and Makino, 1963) have also been shown to influence the occurrence of secondary constrictions. In addition, Hsu et al. (1967) found a difference in the distribution of secondary constrictions between lung fibroblasts and bone marrow cells of *Tamiascurus hudsonicus*. Hence the variability in the expression of secondary constrictions may limit their value in cytotaxonomic studies.

Secondary constrictions have usually been equated with NORs. In situ DNA/RNA hybridization studies have shown that the NOR is the site of the ribosomal RNA cistrons (Pardue et al., 1970; Pardue, 1974; Henderson et al., 1972, 1974; Pardue and Hsu, 1975). However, such hybridization studies have also demonstrated that not all secondary constrictions correspond to NORs (Hsu et al., 1975). Thus secondary constrictions of metaphase chromosomes cannot be unequivocally identified as NORs. When the Ag-As technique was applied to the chromosomes of four species of *Bufo,* silver was preferentially deposited in a prominent secondary constriction located on a single pair of chromosomes in each species. The ribosomal genes appear to be concentrated at a single major site in the genome of these toads such as Hsu et al. (1975) found in several mammals. Hsu et al. (1975) considered the single, long NOR as the more ancestral type in terms of distribution of ribosomal cistrons.

The karyotypes of all four species of *Bufo* were very similar. However, the location of the NOR on chromosome 7 of *B. marinus* allowed its karyotype to be readily distinguished from that of *B. americanus, B. woodhousei* or *B. valliceps* in which the NOR occurred on chromosome 1. *Bufo marinus* is believed to have arisen in South America and later dispersed northward to the southern United States whereas *B. americanus, B. woodhousei* and *B. valliceps* are North American species. The long period of isolation of the North American species from those of South America would have allowed for independent karyotypic evolution and could account for the difference in the location of the NORs.

NORs are often located within heterochromatic segments of chromosomes. Volpe and Gebhardt (1968) reported that the NOR of *B. marinus* occurs within a heterochromatic segment on the short arm of chromosome 7. C-banding of the chromosomes of *B. w. fowleri* indicates that the NOR of this species lies within the centromeric heterochromatin of chromosome 1 (Mahan and Beck, 1977). Hsu et al. (1975) have pointed out that the location of NORs in heterochromatin may simply be due to the fact that they are positioned close to the centromere of chromosomes.

Some specimens of *B. w. woodhousei* exhibited NORs of unequal length. This may reflect variation within different NORs in a cell such as has been observed in other amphibians (Miller and Brown, 1969; MacGregor et al., 1977). Ward (1977) observed NORs of unequal length in *Rana blairi* and concluded that the difference could be due either to deletion or duplication of ribosomal cistrons. This would be reflected as a change in the length of the NOR as seen with the Ag-As technique.

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LITERATURE CITED

BOGART, J. P. 1972. Karyotypes, p. 171–195. *In*: Evolution in the genus *Bufo*. W. F. Blair (ed.). Univ. Texas Press, Austin.

BRUERE, A. N., AND R. D. MCLAREN. 1967. The idiogram of the sheep with particular reference to secondary constrictions. Can. J. Genet. Cytol. 9:543–553.

CALLAN, H. G. 1966. Chromosomes and nucleoli of the axolotl, *Ambystoma mexicanum*. J. Cell Sci. 1:85–108.

COLE, C. J., C. H. LOWE AND J. W. WRIGHT. 1968. Karyotypes of eight species of toads (Genus *Bufo*) in North America. Copeia 1968:96–100.

GOODPASTURE, C., AND S. E. BLOOM. 1975. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma 53:37–50.

HAERTEL, J. D., A. OWEZARZAK AND R. M. STORM. 1974. A comparative study of the chromosomes from five species of the genus *Rana* (Amphibia: Salientia). Copeia 1974:109–114.

HENDERSON, A. S., D. WARBURTON AND K. C. ATWOOD. 1972. Location of ribosomal DNA in the human chromosome complement. Proc. Nat. Acad. Sci. (U.S.) 69:3394–3398.

———, E. M. EICHER, M. T. YU AND K. C. ATWOOD. 1974. The chromosomal location of ribosomal DNA in the mouse. Chromosoma 49:155–160.

HENNEN, S. 1964. The karyotype of *Rana sylvatica* and its comparison with the karyotype of *Rana pipiens*. J. Hered. 55:124–128.

HSU, T. C., B. R. BRINKLEY AND F. E. ARRIGHI. 1967. The structure and behavior of the nucleolar organizer in mammalian cells. Chromosoma 23:137–153.

———, S. E. SPIRITO AND M. L. PARDUE. 1975. Distribution of 18s + 28s ribosomal genes in mammalian genomes. Chromosoma 53:25–36.

MACGREGOR, H. C., M. VLAD AND L. BARNETT. 1977. An investigation of some problems concerning nucleolus organizers in salamanders. Chromosoma 59:283–299.

MAHAN, J. T., AND M. L. BECK. 1977. A karyotypic study of *Bufo woodhousei fowleri*. J. Tenn. Acad. Sci. 52:73.

MILLER, L., AND D. D. BROWN. 1969. Variation in the activity of nucleolus organizers and their ribosomal gene content. Chromosoma 28:430–444.

MORESCALCHI, A., AND G. GARGUILO. 1968. Su alcune relazioni cariologiche del genero *Bufo* (Amphibia Salienta). Rend. Acc. Sc. Fis. Mat. (Naples) 35:117–120.

PALMER, C. G., AND S. FUNDERBURK. 1965. Secondary constrictions in human chromosomes. Cytogenetics 4:261–276.

PARDUE, M. L. 1974. Localization of repeated DNA sequences in *Xenopus* chromosomes. Cold Spr. Harb. Symp. Quant. Biol. 38:475–482.

———, S. A. GERBI, R. A. ECKHARDT AND J. G. GALL. 1970. Cytological localization of DNA complementary to ribosomal RNA in polytene chromosomes of Diptera. Chromosoma 29:268–290.

———, AND T. C. HSU. 1975. Locations of 18s and 28s ribosomal genes in the chromosomes of the Indian Muntjac. J. Cell Biol. 64:251–254.

ROBINSON, E. S., AND E. M. STEPHENSON. 1967. A karyological study of cultured cells of *Limnodynaster peroni* (Anura: Leptodacylidae). Cytologia 32:200–207.

RUDAK, E., AND H. G. CALLAN. 1976. Differential staining and chromatin packing of the mitotic chromosomes of the newt *Triturus cristatus*. Chromosoma 56:349–362.

SASAKI, M. S., AND S. MAKINO. 1963. The demonstration of secondary constrictions in human chromosomes by means of a new technique. Amer. J. Hum. Genet. 15:24–33.

SETO, T. 1965. Cytogenetic studies in lower vertebrates. II. Karyological studies of several species of frogs (Ranidae). Cytologia 30:437–446.

VOLPE, E. P., AND B. M. GEBHARDT. 1968. Somatic chromosomes of the marine toad *Bufo marinus* (Linne). Copeia 1968:570–576.

WARD, O. G. 1977. Dimorphic nucleolar organizer regions in the frog *Rana blairi*. Can. J. Genet. Cytol. 19:51–57.

WASSERMAN, A. O., AND J. P. BOGART. 1968. Chromosomes of two species of spadefoot toads (Genus *Scaphiopus*) and their hybrids. Copeia 1968:303–306.

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