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ORIGINAL ARTICLE

An optimized protocol for obtaining mitotic chromosomes from cultured reptilian lymphocytes

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Abstract We provide an efficient protocol for obtaining mitotic chromosomes with well-defined morphology in 17 different taxa of the class Reptilia. We also show that there is no need for adjustments among taxa and no need to sacrifice the animals studied.

Keywords Neotropical reptiles · In vitro cytogenetical technique · Optimized protocol

Introduction

Cytogenetics and chromosomal evolution have not been studied in as much depth in reptiles as in other groups of vertebrates. The majority of studies have been restricted to karyotypic descriptions with conventional staining; very few studies have used chromosome banding techniques such as C band, NORs, G band [e.g., 2, 5, 10, 14, 15, 17–20]. Chromosomal banding is fundamental for understanding the chromosomal evolution of a group. The low quantity of more refined cytogenetic data in this group, may be due to the difficulty in obtaining good-quality mitotic chromosomes [1, 11]. Among the difficulties are organism handling and maintenance in captivity, but mainly the low mitotic yield due to the slow metabolism in reptiles [7, 13, 21]. Furthermore, techniques that include the use of mitotic fuse inhibitors, such as colchicine in vivo, or that require the animal's death, are impracticable when dealing with medium and large-sized animals such as members of the Boidae family and crocodilians, captive animals (e.g., from zoos and breeders), and threatened taxa.

One of the techniques used in obtaining mitotic chromosomes in reptiles is lymphocyte in vitro culture, which has several variations regarding blood sampling, incubation time and temperature, and reagent concentration. Some studies with snakes used protocols with cardiac puncture for blood sampling, which sometimes require death of the organism [3, 4], which is not desired. Fantin and Monjeló [7] and Noleto et al. [16] obtained chromosomal preparations from blood sampled from the femoral vein of turtles and used the protocol for fish lymphocyte culture described by Fenocchio and Bertollo [8]. However, this protocol was not replicable in other groups of reptiles since it required modifications of the incubation temperature, hypotonic treatment time, and a minimum of 2 mL of blood; amount of blood that is not possible to collect without the death of the animal in some small species of lizards and snakes.

In the majority of lizard studies, mitotic chromosomes were obtained in vivo from the bone marrow according to the protocol of Ford and Hamerton [9]. However, this protocol requires the death of the animal, and it is not always possible to obtain sufficient marrow from small-sized animals. Mitotic chromosomes can be obtained from

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crocodilian blood samples according to the protocols of Cohen and Gans [6] and King et al. [12]. However, modifications are required in the incubation time for different species [1], and the quality of the preparations is not adequate since the morphology of some chromosomes is not well defined.

Since there are a variety of protocols for obtaining chromosomes in reptiles, each with its own limitations, we employed previously described protocols [1, 4, 8] to optimize the lymphocyte culture technique for different groups of the class Reptilia.

Materials and methods

We sampled specimens of Testudines, Squamata, and Crocodylia, and we collected blood using syringes with heparin sodium in proportion of 0.1 mL to each 1 mL of blood, with needles of sizes of 0.38/13, 0.45/13 and 0.55/20 mm. The blood was then immediately transferred to heparinized vacutainer tubes. Blood from snakes, crocodilians/lizards and turtles was obtained from the dorsal vein, the occipital sinus, and the femoral vein, respectively. We obtained a minimum of 800 µL of blood from each sample. The vacutainer tube was left resting in a vertical position, at room temperature, for 30–60 min until the blood plasma separated. Next, we added 500 µL of the interstitial layer, between the decanted red blood cells and the plasma, to a complete medium for culture (Cultilab®, Faz. Santa Candida, 13087-567/Campinas, SP/Brazil) made up of RPMI 1640 medium, HEPES, fetal bovine serum, antibiotics, and phytohemagglutinin or a mix in a 15 mL tube made up of: 3.75 mL of RPMI 1640 medium, 1.0 mL of fetal bovine serum 20 %, 0.5 mL of antibiotics, 0.2 mL of phytohemagglutinin and some drops of hepes for balancing pH to neutral (Sigma® or Cultilab®), resulting in a total volume of 5.45 mL for each sample. Later, in all samples, an additional 100 µL of phytohemagglutinin was added to the medium, and the tube was gently agitated. The material was kept in an incubator at 29 °C for 96 h and gently homogenized every 24 h.

At 96 h of incubation, 500 µL of colcemid 0.025 % was added, and the tubes were gently agitated and maintained at 29 °C for 50 min. Next, the material was transferred to a 15 mL tube and centrifuged for 10 min at 1200 rpm. The supernatant was discarded, and 10 mL of hypotonic KCl 0.075 M solution was added. The material was then resuspended and kept in the incubator at 37 °C for 50 min. After incubating, 100 µL of Carnoy's solution (3 methanol:1 acetic acid) was added, and the material was homogenized and centrifuged for 10 min at 1200 rpm. Next, the supernatant was discarded. Then, 10 mL of the

Table 1 Taxa of Reptilia analyzed; quantity and diploid number found

Suborder	Species/subspecies	N_1	N_2	Average number of metaphases	2n
Serpentes	*Boa constrictor constrictor*	18	18	+++	36
	Boa constrictor amarali	10	10	+++	36
	Boa constrictor	1	1	++	36
	Corallus sp.	7	7	+	40
	Eunectes murinus	10	10	+++	36
	Eunectes sp.	1	1	++++	36
	Epicrates crassus	3	3	++	36
	Epicrates sp. 1	2	2	+	36
	Epicrates sp.	2	2	+	36
	Spilotes sp.	1	1	++	36
Lacertilia	*Ameiva ameiva ameiva*	1	1	+++	52
Cryptodira	*Rhinoclemmys punctularia*	2	2	+++	56
Pleurodira	*Chelus fimbriata*	5	5	+++	50
	Phrynops geoffroanus	16	16	++++	58
	Mesoclemmys gibba	6	6	++++	60
	Mesoclemmys sp. 1	1	1	++	52
	Mesoclemmys sp.	5	5	++++	42
	Peltocephalus dumerilianus	5	5	++	26
Caimaninae	*Caiman crocodilus*	2	2	+++	42
	Paleosuchus trigonatus	2	2	++	42

N_1, number of samples analyzed; N_2, number of samples with satisfactory results. Between 5 and 10 metaphases per slide (+); between 11 and 20 metaphases per slide (++); between 21 and 30 metaphases per slide (+++); 31 or more metaphases per slide (++++)

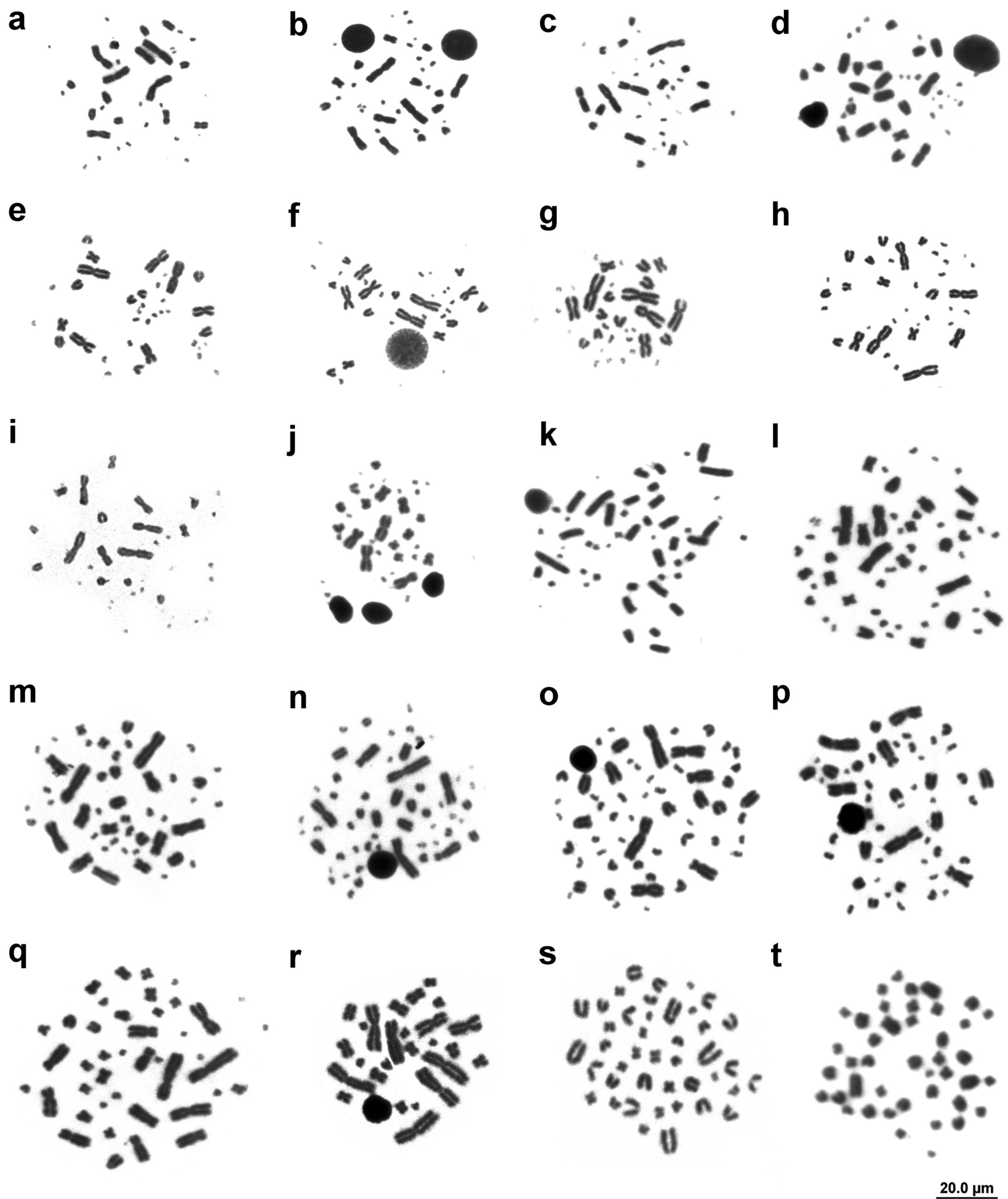

Fig. 1 Metaphases of different taxa of Reptilia analyzed. **a** *Boa constrictor constrictor*, **b** *Boa constrictor amarali*, **c** *Boa constrictor*, **d** *Corallus* sp., **e** *Eunectes murinus*, **f** *Eunectes* sp., **g** *Epicrates crassus*, **h** *Epicrates* sp. 1, **i** *Epicrates* sp. 2, **j** *Spilotes* sp., **k** *Ameiva ameiva ameiva*, **l** *Rhinoclemmys punctularia*, **m** *Chelus fimbriata*, **n** *Phrynops geoffroanus*, **o** *Mesoclemmys gibba*, **p** *Mesoclemmys* sp. 1, **q** *Mesoclemmys* sp. 2, **r** *Peltocephalus dumerilianus*, **s** *Caiman crocodilus*, **t** *Paleosuchus trigonatus*

Carnoy's solution at −10 °C was added, and the material was re-suspended until a homogenous solution was obtained. The material was again centrifuged for 10 min at 1200 rpm, and the supernatant was discarded. Finally, 5 mL of the Carnoy's solution was added, and the material re-suspended and centrifuged for 10 min at 1200 rpm; this step was repeated twice, always discarding the supernatant. After the last centrifuge, the supernatant was discarded, and between 1 and 1.5 mL of Carnoy's solution was added. This chromosome preparation was kept in a 1.5 mL microtube at −4 °C until slide preparation. The clean glass slides were immersed in distilled water at 48 °C and removed after 5 min. This procedure ensured that a film of water was retained on the surfaces of the slides. A 40 µL aliquot of the chromosome preparation was dripped over different regions of each slide and left to air dry. The slides were then stained with 5 % Giemsa diluted in pH 6.8 phosphate buffer for 10 min and analyzed under a microscope.

Results and discussion

Our methodology was effective at obtaining mitotic chromosomes in reptiles of different metabolic rates from different orders; all of our samples presented satisfactory results in regards to the presence of metaphases and chromosome quality. The quantity of metaphases per slide varied among taxa (Table 1). However, even among the specimens with few metaphases, the quality of the material was maintained (i.e., distended chromosomes and well-defined morphology; Fig. 1), allowing a better analysis of the macro- and micro-chromosomes, which are present in most species of this group of vertebrates.

The quality of the chromosome preparations obtained in the current study also allows the use of techniques such as 'C' and 'G' banding, nucleolus organizer region, and physical mapping of DNA sequences without loss of morphological characteristics in the chromosomes [unpublished data]. Furthermore, the quality and degree of chromosome compaction for the taxa that we analyzed were improved relative to the findings of studies of crocodilians [1] and turtles [11], where it was possible to note difference in the quality of the chromosome preparations. The quality of our preparations also exceeded those of a study of turtles from the family Podocnemididae [7].

Since it is possible to maintain different temperatures (medium incubation and hypotonic treatment) in the field and disinfected the place of incubation with alcohol 70 %, this protocol may be easily repeated in long-term excursions. As an example, we highlight the *Eunectes murinus*, *Ameiva ameiva ameiva*, *Boa constrictor constrictor*, *Corallus* sp. and *Caiman crocodilus* preparations that were obtained in field, and all of them presented satisfactory quality with well-spread and distended chromosomes (Fig. 1; Table 1).

Conclusions

The current protocol is effective and is presented largely due to its repeatability with different species of Reptilia without the need for methodological adjustments for different taxa. Furthermore, it is expected that this methodology will enable an increase in cytogenetic studies with taxa of the class Reptilia; such studies have been rare for some groups.

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Authors' contributions The authors have made the following declarations about their contributions. P.V. and L.R. conceived, designed and directed the experiments. P.V., E.F., M.C.G., L.R., R.C.V., T.L., V.T.C. performed the experiments. P.V. wrote the first draft and all coauthors contributed in reviewing the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

All the steps of this study were in accordance with the committee of animal ethics National Institute of Amazonia Research.

Conflict of interest All authors declare that they have no conflict of interest.

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