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Use of peripheral blood lymphocyte culture in the karyological analysis of Indian freshwater turtles, *Lissemys punctata* and *Geoclemys hamiltoni*

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A simple, efficient and economic method has been optimized for *in vitro* culture of peripheral blood lymphocytes from two freshwater turtles, *Lissemys punctata* (Trionychidae) and *Geoclemys hamiltoni* (Batagurinae), for various parameters like culture media, mitogen concentration, mitotic index, culture volume, incubation time, humidity, duration of culture or mitotic arrest, etc. The most optimal condition for good mitotic index was obtained when the lymphocytes were cultured at 28°C in a common RPMI-1640 medium with L-glutamine but without $NaHCO_3$, 10% FCS, PHA-P as mitogen (10 µg/ml), streptomycin (100 µg/ml), penicillin (50 µg/ml) and incubated for a period of 84 h. The mitotic arrest was made for 10 to 12 h with colcemid (0.2 µg/ml) after 72 h of setting the culture. The non-banded mitotic chromosomes of both the species were characterized. The diploid chromosome (2N) numbers were found varying in the two species, being 66 in *L. punctata*, and 52 in *G. hamiltoni*. The difference in the chromosome numbers in the two species was basically in the telocentric- and micro-chromosomes, both being higher in *L. punctata* than in *G. hamiltoni*. Except for some of the large or macrochromosomes most others appeared species-specific, indicating their distinct evolutionary lineage among Chelonians.

Keywords: Chelonia, *Geoclemys hamiltoni*, *Lissemys punctata*, lymphocyte culture, turtle karyotype,

FOR studies on cytotaxonomy or chromosomal polymorphisms in endangered wildlife species, the source or number of animals becomes limiting. The classical methods of chromosome preparation from spleen, kidney or intestine[1,2] or cell culture of heart and skin fibroblast[2] involves killing or surgery, often being unsafe for survival of the animal, traumatic and unethical. In such cases, karyotype analysis from cultured blood lymphocytes without sacrificing the animal is most desirable and useful, particularly in reptiles whose body temperature varies with environment and exhibits a slow cell-division cycle. Few reports are available on lymphocyte cultures of reptilian species such as *Alligator mississippensis*[3,4] and *Trachemys scripta*[5]. Not all the cell-culture parameters optimized in the above methods are applicable to Indian species because of their differential adaptability, variation in preferred body temperature, lymphocyte population and immunological responses against mitogen stimulation.

Cytogenetic identification is one of the important parameters for the conservation of organisms in their natural habitat. For this purpose, the first level of genome analysis involves karyotyping of mitotic chromosomes to know the organization of the organismal genome at the cytological level. Turtles and tortoises are the most distinctive reptilians that have retained their basic features since Triassic period. From the viewpoint of cytotaxonomy and molecular cytogenetics, this is the most neglected group of reptiles. Only limited published data are available on the morphology of chromosomes and karyological trends of evolution in turtles[6–9]. The other most significant characteristic of freshwater turtles that tempt detailed cytogenetic study on the mitotic or meiotic chromosomes is their recognition as indicators of water quality of an aquatic body. They can, therefore, be used to assess genotoxicity induced by potential toxicants discharged in the water bodies following cytogenetic analysis of chromosomal aberration[10]. The present report is a successful attempt on an *in vitro* culture of peripheral blood lymphocytes for the preparation of mitotic chromosomes from two species of freshwater turtles, *Lissemys punctata* and *Geoclemys hamiltoni*, for studies on cytotaxonomy and chromosomal polymorphism. The non-banded karyotypes of *L. punctata* and *G. hamiltoni* prepared from short-term lymphocyte cultures revealed species-specific variations in their chromosome complements, mostly in telocentric and morphologically indistinct micro-chromosomes.

Experiments were carried out on young adult individuals of soft-shell turtle *L. punctata*, which were collected

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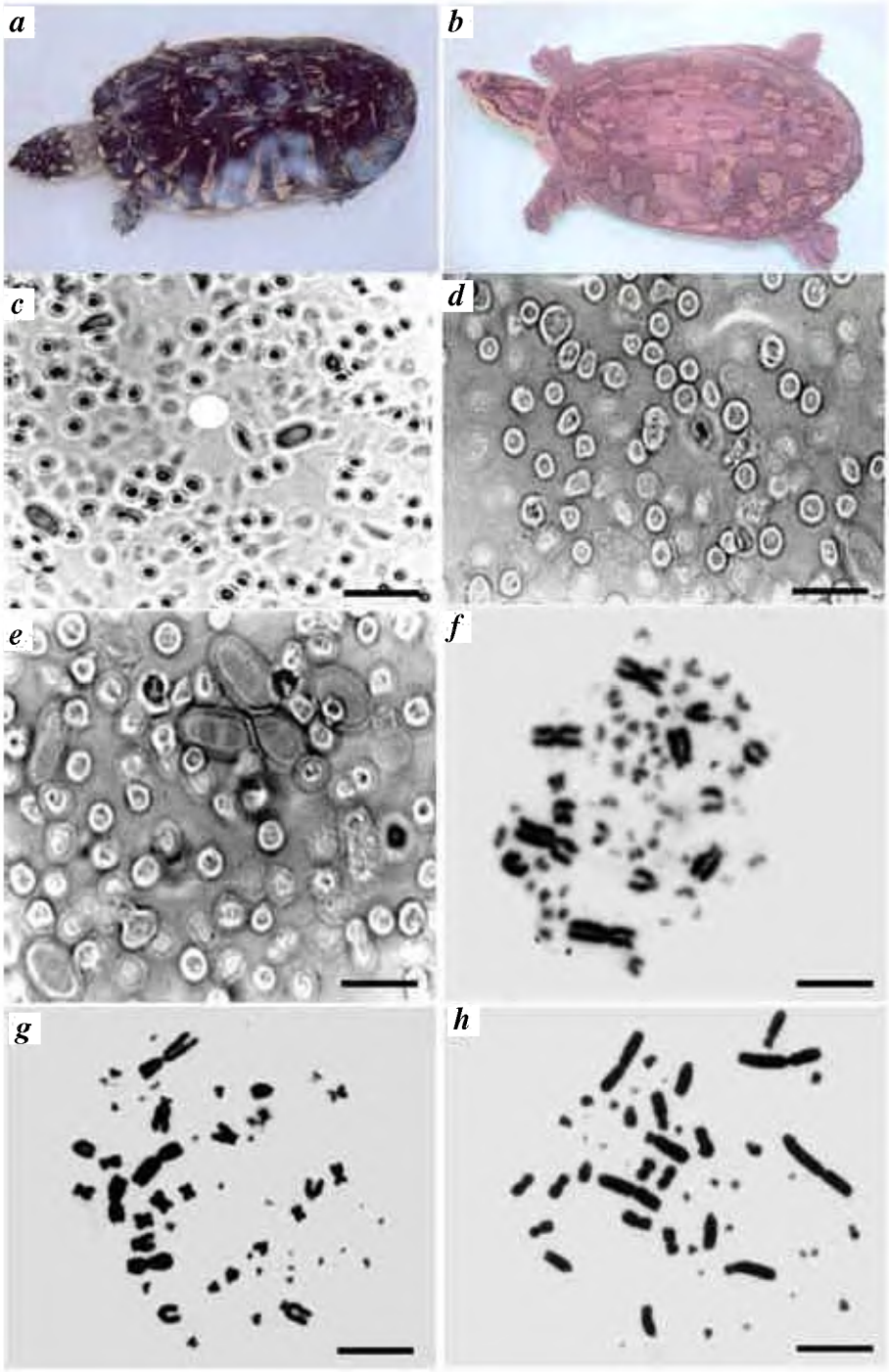

Figure 1. ***a***, ***b***, Morphological features (shell) of freshwater Chelonian species. *Geoclemys hamiltoni* (***a***) and *Lissemys punctata* (***b***); ***c–e***, Phase contrast images of lymphocytes after seeding in medium showing morphology and cell population (***c***) (magnification 50X; bar represents 80 μm); After 48 h of culture (***d***) (magnification 200X; bar represents 20 μm); After 84 h of culture (***e***) in medium (magnification 200X; bar represents 20 μm); ***f***, Metaphase chromosome of *L. punctata* (magnification 500X; bar represents 10 μm) and ***g***, ***h***, Metaphase chromosomes of *G. hamiltoni* (magnification 500X; bar represents 10 μm).

from ponds located in Gwalior–Chambal region with the help of local fishermen. *G. hamiltoni* was collected from Ganga river at Narora, Uttar Pradesh (Figure 1 *a*, *b*). They were reared in an artificial pond, especially constructed for them, and partially mimicking their natural habitat. Animals were fed on natural diet of freshwater fishes such as *Labeo rohita*, *Catla catla*, *Notopterus notopterus*, live earthworms and snails. The animal care procedures followed in this study were as recommended by animal ethical committee of Jiwaji University and with prior

Table 1. Components of short-term culture of peripheral blood lymphocytes of freshwater turtle

Component	Culture volume	
	10 ml	20 ml
RPMI-1640 w/o $NaHCO_3$ with L-glutamine (Hyclone, Utah, USA)	7.7 ml	15.4 ml
Streptomycin (10 mg/ml) (Himedia, Mumbai, India)	100 µl	200 µl
Penicillin (10,000 IU/ml) (Himedia)	100 µl	200 µl
PHA-P (1 mg/ml) (Sigma)	100 µl	200 µl
Foetal calf serum (heat-inactivated; Hyclone)	1 ml	2 ml
Lymphocytes with serum (separated from 5 ml whole blood)	1 ml	2 ml

permission of the Department of Forests, Government of Madhya Pradesh.

Hind limbs were cleaned with distilled water-soaked cotton and after complete drying swabbed twice with absolute ethanol. About 5 ml blood was withdrawn from the femoral vein with the help of 5 ml syringe equipped with 25G 5/8-inch needle preflushed with sodium heparin. The blood was immediately transferred to sterilized microcentrifuge tubes containing heparin (20 IU; the final volume being 100 µl per ml of blood) and stored at 28°C for 2 h. After blood collection the skin was again swabbed with absolute ethanol and medicated with Soframycin cream to prevent microbial infection. The animal was left on the sand to recover for sometime before it was transferred to water. Various cell-types such as RBC and lymphocytes were differentiated in microcentrifuge tube according to their density. The upper layer lymphocytes were transferred to a sterilized microcentrifuge tube, incubated at 28°C for complete separation of lymphocytes from lower layer, mainly enriched in RBCs.

In a 30 ml Borosil tissue culture vial, 7.7 ml of RPMI 1640 culture medium was taken. The pH of the medium was brought to 7.2 by blowing CO_2 through the mouth with the help of a sterilized Borosil glass pipette. All other components were added serially as mentioned in Table 1. These steps were carried out in sterile condition under laminar flow hood to avoid any microbial infection. The culture was incubated in a humidified incubator set at 28°C. The humidity was maintained with sterilized distilled water filled in pie dishes and placed in the lowest chamber of the air-circulating incubator. The culture vial cap was loosened and placed at an inclination angle of 45° for proper gas exchange, increasing surface area and to avoid loss of medium due to evaporation and fluctuation in the pH of culture medium throughout the incubation period. Since isolated lymphocytes are highly sensitive to change in culture volume, sufficient medium (at least 10 ml) was taken for seeding of cells. The culture was shaken gently every morning and evening to break the cell clumps. After 72 h of incubation, colcemide was added to a final concentration of 0.2 µg/ml and the culture was harvested after 82–84 h for good mitotic count[5]. The culture medium was transferred to a 15 ml sterilized polypropylene tube and centrifuged at 4000 rpm for 5 min. After discarding the supernatant, the cell pellet was resuspended in the hypotonic solution (1 ml of prewarmed 0.50% KCl) and incubated at 28°C for 30 min. At the end of hypotonic treatment, a few drops of fixative (one part glacial acetic acid and three parts absolute methanol) was added to the hypotonic solution to prefix the cells, followed by centrifugation at 4000 rpm for 5 min. Again, after discarding the supernatant, the cell pellet was resuspended in 1 ml freshly prepared acetomethanol following gentle agitation. The last step (fixation) was repeated twice for proper fixation of cells. The cells were finally suspended in 0.5 ml fixative for chromosome preparation. Chromosome were prepared by splash technique[11], stained with 0.01% Giemsa stain prepared in Sorenson's buffer[5], differentiated in distilled water, air-dried and mounted in DPX for microscopic observation.

Chromosomes from at least 20 metaphase plates were counted for each turtle and the best mitotic plates were photographed under a Nikon Labophot (Nikon, Japan) photomicroscope. Chromosomes from both *L. punctata* and *G. hamiltoni* were identified according to Bickham[2] and arranged in three groups: (i) Group A, consisting of large size macrochromosomes, (ii) Group B consisting of medium size macrochromosomes and (iii) Group C including all the microchromosomes whose centromeric positions were not clearly identifiable under light microscope.

Our method of culturing peripheral blood lymphocytes of turtles for cytogenetic studies was found highly economic, simple and efficient. There have been a few successful attempts in this regard involving American alligators and European Chelonians. The Indian species of freshwater turtles are unique compared to the European species regarding various characteristics, such as preferred body temperature, specific morphological features like lack of protruding tail that may facilitate easy withdrawal of blood, etc. In *Lissemys* and *Geoclemys*, the tail is short and blood collection from dorsal cervical sinus, coccygeal vein and scapula vein or brachial artery, as described for European and marine species such as *Trachemys scripta*, *Chelonia mydas* and *Geochelone*[5,12,13], is not convenient. They cannot be held firmly by any recommended device or manually. Further, due to their aggressive behaviour, blood collection from dorsal cervical sinus is another problem

Table 2. Chromosome (karyotype) characteristics of *Lissemys punctata* and *Geoclemys hamiltoni*

Turtle species	Submetacentric chromosomes	Metacentric chromosomes	Acrocentric chromosomes	Telocentric chromosomes	Unidentified microchromosomes
L. punctata (2*N* = 66)	4	0	2	26	34
G. hamiltoni (2*N* = 52)	4	14	4	6	24

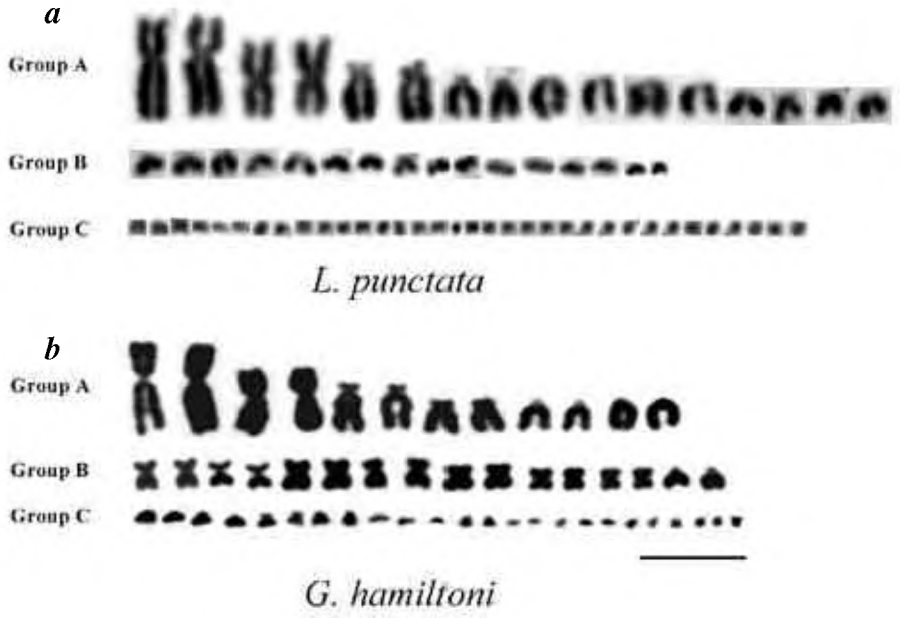

Figure 2. Non-banded karyotype of *L. punctata* (***a***) and *G. hamiltoni* (***b***). (magnification 500X; bar represents 10 μm).

with live animals and, if performed, requires anesthesia. The method of blood collection described in this study was, therefore, found more efficient and safe for the animal also. It is easy to hold healthy 4–5 kg animals from their hind legs for drawing blood (up to 10 ml) without much difficulty and causing any harm to the animal.

The optimized conditions in this study have yielded consistently good results with reasonably good mitotic index (MI) of about 15–20 metaphase plates per slide. Throughout the cell culture period, cellular morphology was monitored for mortality. In general, except for those lymphocytes which were more sensitive to change in the physiological environment, a good number of cells were found surviving (Figure 1 *c–e*). Though the number of dividing cells was few due to much slower rate of cell cycling in turtle compared to mammalian cells, sufficient number of well-spread mitotic plates was obtained (Figure 1 *f–h*), good enough for karyotype analysis. Our method is distinct in many respects from those described for mammals, including humans and also for reptiles[5,14]. Among the major differences between reptiles and mammals are the presence of nucleated RBCs in the reptiles, the preferred body temperature, cell-cycle periods and mitogenic responses of mammalian and reptilian lymphocytes that require optimization of various culture parameters[5,15,16]. The culture technique we have optimized for freshwater turtles can be applied to other turtle species also with certain modifications in essential parameters like incubation temperature, incubation period, serum concentration, mitogen, etc. In comparison to an earlier study in *T. scripta*[5], where standard conditions of culture yielded an MI of 1.3 ± 0.27, this method produced a similar MI of 1.35 ± 0.24 (MI% ± SD%), with minimal essential requirements. Therefore, the present attempt is significant since there is a need of developing a viable and economic protocol for cell culture in freshwater turtles without harming the animal, particularly for a moderately equipped laboratory working on cytotaxonomy, genotoxicity or molecular and cytogenetic analysis of turtle chromosomes.

The non-banded karyotype prepared from *L. punctata* revealed diploid (2*N*) chromosome number to be 66 (Table 2). In Group A, one pair of large submetacentric, one pair of metacentric, one pair of acrocentric macrochromosomes and five pairs of medium size telocentric macrochromosomes have been included. Group B consists of eight pairs of small size telocentric chromosomes, while Group C includes all 34 microchromosomes whose centromeric positions are not clearly recognizable. None of the chromosomes appeared metacentric. In comparison to *L. punctata*, *G. hamiltoni* showed only 52 (2*N*) in its diploid complement with two pairs of large submetacentric macrochromosomes, two pairs of acrocentric macrochro-

mosomes and two pairs of telocentric macrochromosomes in Group A (Figure 2). Group B consists of seven pairs of small-size metacentric macrochromosomes and one pair of small acrocentric chromosomes, while Group C has 24 unidentified microchromosomes (Table 2). Both species showed strong similarities in chromosome type and number with their respective family members, as reported earlier[6,10,17]. This study further strengthens the highly conserved nature of karyological trend over different geographical localities in Trionychidae and Batagurinae.

Compared to soft-shell turtles (Trionychidae), all family members of hard-shell turtles have relatively lower number of microchromosomes in their diploid complements, which is a direct reflection of evolutionary course and adaptive radiation of Chelonian divergence. The mechanisms responsible for the reduction of microchromosomes in the long evolutionary history of Chelonians, from the most ancient form, Trionychidae to modern members, e.g. hard shell turtles, could be the loss in numbers, translocation of microchromosomes to acrocentric chromosomes and formation of metacentric chromosomes[2]. A similar mechanism might have been involved in the reduction of telocentric chromosomes also. Bickham[2] reported the karyotypic differences among two subfamilies of hard shell turtles with the diploid chromosome number being 50 (2*N*), in the New World genera of family Emydidae (subfamily Emydinae) and 52 (2*N*) in the members of Old World genera of subfamily Batagurinae. The karyotypic differences between these two subfamilies are mainly in the presence of an extra pair of small metacentric chromosomes in the Batagurinae, which may have been lost entirely or broken up and translocated to other chromosomes in the Emydinae[3].

Molecular and cytogenetic evidences suggest that the vertebrate microchromosomes mainly contain GC-rich repetitive DNA and may be more gene-dense than macrochromosomes[18]. Therefore, the banding procedure does not differentiate the reptilian microchromosomes well, but macrochromosomes do so with all the methods[19,20]. Presence of microchromosome-specific repetitive sequences was also reported in birds (Struthioniformes and Galliformes), suggesting that both turtles and birds have retained some similarity in their chromosomes or karyotype structures during evolution from some common primitive ancestors[21].

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