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BRIEF REPORT

A simple non-invasive protocol to establish primary cell lines from tail and toe explants for cytogenetic studies in Australian dragon lizards (Squamata: Agamidae)

Tariq Ezaz · Denis O'Meally · Alexander E. Quinn · Stephen D. Sarre · Arthur Georges · Jennifer A. Marshall Graves

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Abstract Primary cell lines were established from cultures of tail and toe clips of five species of Australian dragon lizards: *Tympanocryptis pinguicolla*, *Tympanocryptis* sp., *Ctenophorus fordi*, *Amphibolurus norrisi* and *Pogona vitticeps*. The start of exponential cell growth ranged from 1 to 5 weeks. Cultures from all specimens had fibroblastic morphology. Cell lines were propagated continuously up to ten passages, cryopreserved and recovered successfully. We found no reduction in cell viability after short term (<6 months) storage at −80 °C. Mitotic metaphase chromosomes were harvested from these cell lines and used in differential staining, banding and fluorescent in situ hybridisation. Cell lines maintained normal diploidy in all species. This study reports a simple non-invasive method for establishing primary cell lines from Australian dragon lizards without sacrifice. The method is likely to be applicable to a range of species. Such cell lines provide a virtually unlimited source of material for cytogenetic, evolutionary and genomic studies.

T. Ezaz · D. O'Meally · J. A. Marshall Graves
Comparative Genomics Group, Research School of Biological Sciences, The Australian National University, GPO Box 475, Canberra, ACT 2601, Australia

T. Ezaz (✉) · A. E. Quinn · S. D. Sarre · A. Georges
Institute for Applied Ecology, University of Canberra, Building 3, Canberra, ACT 2601, Australia
e-mail: Tariq.Ezaz@canberra.edu.au

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Introduction

Australian dragon lizards (Agamidae) are an ideal group in which to study sex chromosome evolution because of their recent radiation from an Asian ancestor (~25 million years ago; Hugall et al. 2008) and because there appears to have been multiple transitions between genotypic and temperature-dependent sex determination (Ezaz et al. 2009; Harlow 2004; Sarre et al. 2004). There are about 70 species in 13 genera (Cogger 2000). Although karyotypes have been described for many species, about 60% remain unexplored (Olmo 2005). In our laboratory, we routinely establish short term blood culture and primary fibroblast cell lines from internal connective tissue for larger species of agamid. From these we harvest chromosomes and interphase nuclei, and extract nucleic acids (DNA, RNA) for molecular and cytogenetic studies. However, some species cannot be sacrificed as they are listed as endangered or vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Australia). Many species are too small to obtain sufficient volumes for blood culture and would otherwise require invasive or post mortem sampling.

Explant culture is a routine procedure for establishing fibroblast-like cell lines from various organs,

and it has been optimised in various animals and used in a wide variety of biological experiments (Freshney 2006; Masters and Stacey 2007). Cell lines established from explant culture provide an almost unlimited source of chromosomes, interphase nuclei, DNA, RNA and other biomolecules, which can then be used in a wide variety of genomic and evolutionary studies. However, use of internal organs as explant for the establishment of primary fibroblast cell lines requires euthanizing animals, an option that is often not available in endangered and threatened species. An alternative approach to obtaining metaphase chromosomes is to set up short term blood culture but that too can be difficult in small species where the collection of sufficient blood necessary to establish successful cultures is problematic or when bleeding may lead to infection and stress to animals. A non-invasive approach would clearly be of value in such cases.

Culture of tail and epidermal explants has been reported in one lizard (*Anolis carolinensis* Simpson and Cox 1967); and a turtle (*Chelonia mydas* Mansell et al. 1989); while caudal fin explant has been used to establish primary cell lines in channel catfish (Zhang et al. 1998). However, the majority of reported studies used either embryonic tissues or various organs collected post mortem (see Mansell et al. 1989 and references therein).

In the current study, we developed a non-invasive technique to establish primary fibroblast cell lines from explant culture of tail and toe clips, and optimised culture conditions and cryopreservation of these cells lines. We characterized the cell lines by harvesting metaphase chromosomes and subsequent cytogenetic analysis.

Methods

Animals

Wild animals were collected from various locations across Australia. Mallee dragons (*Ctenophorus fordi*) were collected from Yathong Nature Reserve (32°S 145°E), Norris' dragons (*Amphibolurus norrisi*) were collected from Big Desert National Park (35°S 141°E), grassland earless dragons (*Tympanocryptis pinguicolla*) were collected from Canberra (35°S 149°E), and *Tympanocryptis* sp. (species not determined) were collected from Corella Creek Station (19 21°S × 136 02°E) and central bearded dragons (*Pogona vitticeps)* were collected from Murray-Sunset National Park (34°S 141°E).

Reagents and culture medium

Washing Buffer (WB): 1× PBS (calcium–magnesium free, pH 7.2–7.4 with phenol red). Collection Medium (CME): prepared inside a laminar hood by supplementing plain DMEM (Gibco) with 1 mg/mL kanamycin (Sigma), 40 µg/mL chloramphenicol (Sigma), 60 µg/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco). This mix was sterilized by passing through a 0.2 µm filter (Millipore GP). Subsequently, amphotericin B (Sigma) and tetracycline chloride (Sigma) were added to a final concentration of 20–100 µg/mL, respectively. We have found collection medium can be stored in 50 mL aliquots for up to 6 months at −20 °C. Culture Medium with Antibiotics (CMA): Amniomax C-100 (basal medium and supplement, Gibco), 100 U/mL penicillin G, sodium salt (Gibco), and 100 µg/mL streptomycin sulfate (Gibco). Trypsin Solution (TS): 0.1% w/v in 1× PBS, 0.1% w/v tetrasodium EDTA (Sigma). Freezing Medium (FM): 20% foetal bovine serum (Invitrogen) and 10% DMSO (Sigma) in plain DMEM. Hypotonic Solution (HS): 0.075 mM KCl. Fixative (FIX): 3 parts methanol, 1 part acetic acid.

Tissue collection

The surfaces of the tail tips and toes of adult dragon lizards were sterilized by wiping with gauze soaked in 70% ethanol. Approximately 5–10 mm of the tail tip was collected from each specimen using a sterile scalpel blade (*A. norrisi*, one male, one female; *C. fordi*, three females, *T. pinguicolla*, one male, *Tympanocryptis* sp. one male and *P. vitticeps*, one female). Three millimetres of the fourth toe of the hind foot were collected from a single male *A. norrisi* specimen. Tail and toe clips were immediately transferred to 5 mL of CME and incubated at room temperature (∼25 °C) for 12–24 h.

Cultures

All explants for cultures were set up in an aseptic environment inside a laminar hood using sterilised

equipment. Individual tissue pieces were placed onto a Petri dish and washed twice with 1× WB. These were then transferred to a new Petri dish containing two drops plain DMEM and minced with scalpel blades. Culture flasks were prepared by scratching the inner surface with a scalpel blade (to assist in attachment of explants) and coated with 2 mL of plain DMEM, which aids later spreading of the medium without dislodging explants. Minced tissue pieces were transferred using a Pasteur pipette to a T25 culture flask and left to dry upright, with the lid open, for 30–60 min inside the hood after which 5 mL of CMA were added. Flasks were then transferred to a humidified incubator at 28 °C with 5% CO_2.

Splitting and freezing cultures

Cells were passaged once high density cell growth around primary explants was observed. Each flask was rinsed once with 3 mL 1× WB. Then, following the addition of 1.5 mL of TS, the flask was incubated at RT for 1–2 min or until cells were dislodged from the flask surface (with gentle tapping and observation under a light microscope) and mixed gently with a Pasteur pipette to remove cell clumps. To collect the cells, 5 mL (~3 volumes of TS) of CMA was added to the flask and transferred to a 10 mL tube. The cell suspension was centrifuged at 250*g* in a swivel rotor centrifuge for 5 min and the supernatant discarded, leaving 100–200 μL behind. The resultant cell pellet was then resuspended in 2 mL of CMA and mixed gently by tapping and then transferred to a new flask containing 3 (for T25) or 8 (T75) mL of CMA. Flasks were incubated as described above.

For freezing, a cell pellet was obtained as above, but resuspended in 1.5 mL FM, transferred to a chilled cryovial (Nunc) with appropriate labelling and stored at −80 °C for at least 2 days. For long term archival storage, vials were transferred to a liquid N_2 storage facility. We found cells were viable when stored at −80 °C for up to 6 months.

Chromosome preparations, differential staining, banding, FISH and microscopy

Metaphase chromosomes were harvested as described by Ezaz et al. (2005) with minor modifications. Specifically, cells were grown in culture until there was a high proportion of dividing cells (~50% with 60–70% overall confluence). The dividing cells are normally rounded and appear as doublets. Cell divisions were blocked at metaphase by adding 0.05 μg/mL of Colcemid (Roche) and incubated as described earlier for up to two hours, checking under a microscope every half an hour. When cells respond to colcemid (with high frequency of rounded cells), the medium was discarded, trypsinized, centrifuged and aspirated as described above. The pellet was resuspended gently and 1 mL HS added drop by drop. Another 2 mL of HS was then added slowly, mixed gently and incubated in a 37 °C water bath for 45–60 min. Several drops of freshly prepared chilled fixative were added and the cells centrifuged as before. The supernatant was removed except for 1 mL in which the pellet was resuspended. The cells were fixed by slowly adding 1 mL FIX. Another 1 mL of FIX was added and centrifuged as before. The cell pellet was washed three more times and finally FIX was added on the basis of the size of the pellet. The quality and mitotic index of the cell suspension was examined by dropping cells onto microscope slides and observing under a phase contrast microscope. Fixed cell suspensions were kept at −20 °C for long-term storage. Differential staining (DAPI, CA3, banding (C-banding), FISH with telomeric probe (TTAGGG)n and microscopy were performed as described by Ezaz et al. (2005).

Results and discussion

We have presented techniques to establish, propagate, maintain and archive primary fibroblastic cell lines from toe and tail clip explants of Australian dragon lizards, without the need to sacrifice animals.

Cells started to grow out from the explants within 4–5 days and all primary explants yielded dividing cells. The time required for initiation of exponential cell growth (~40–70% confluency) was variable, ranging from 1 to 5 weeks. In all species, explants produced primary cell lines with fibroblastic morphology (Fig. 1a). Cell lines were maintained and propagated through up to ten passages and cryopreserved successfully. After each passage, 70–80% confluency was reached within 1–3 days (Fig. 1a), after which cells were subcultured at at a 1:1 ratio. Cells were also subcultured at 1:3 and 1:4 ratios and

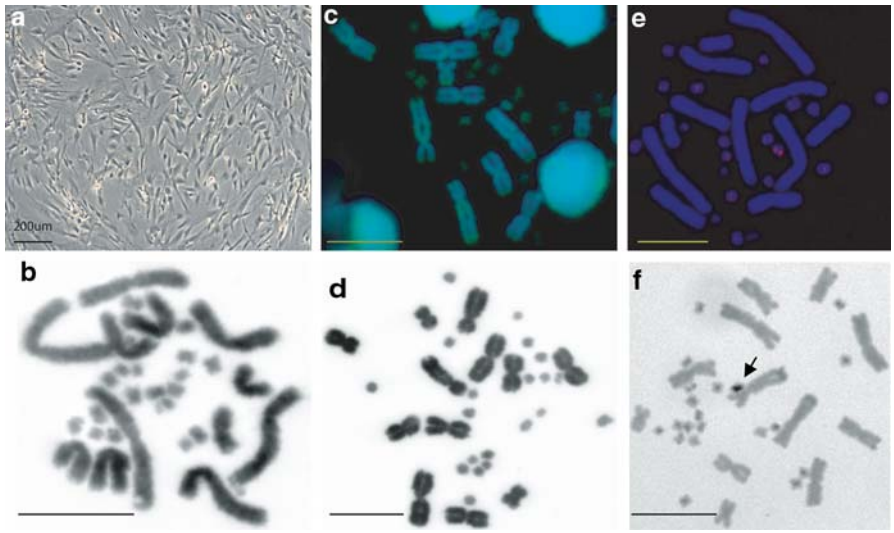

Fig. 1 Examples showing fibroblast cells and chromosomes harvested from tail explants of Australian dragon lizards. **a** fibroblast cell line obtained from tail clip of *T. pinguicolla*; **b** inverted DAPI stained chromosomes in *T. pinguicolla*; **c** merged images of CA3 and DAPI stained chromosomes in *A. norrisi*; **d** inverted DAPI stained images in *C. fordi*; **e** FISH of telomeric probe in *P. vitticeps*; **f** C-banded chromosomes of female *P. vitticeps*, *arrow indicates* C-banded W chromosomes. *Scale bar* represents 10 μm

50–70% confluency was reached within 1–3 weeks. No substantial mortality was observed when subcultured at the lower innoculi. The medium was changed once a week but no significant mortality was observed when the medium was changed only after 2 weeks. We found no reduction in cell viability after short term (<6 months) storage at −80 °C. We found no mortality in response to trypsin in any of our cell lines and no reduction of growth after recovery from cryopreservation.

To optimise culture conditions we varied the composition of the culture medium, incubation temperature and maceration versus enzymatic (collagenization) methods of primary cell isolation (data not shown). We compared explant cultures in 10% DMEM and 1:1 ratio of 10% DMEM and Amniomax to that of cultures with Amniomax only. We found optimum cell growth when cultured in Amniomax only. We incubated cultures at three temperatures: 26, 28 and 30 °C. We observed optimum cell growth in cultures incubated at 28 °C. We have also compared collagenization of explants with the maceration technique described herein. We found high cell mortality as well as frequent bacterial and fungal infections when collagenization was used. We observed no fungal or bacterial infection when our maceration technique was used.

Metaphase chromosome preparations were obtained from all cell lines reported here (Fig. 1b–f). The diploid chromosomes in Australian dragon lizards are highly conserved with the majority of species (including the five species in this study) having a chromosome complement of $2n = 32$ comprising 10 macro-chromosomes and 12 micro-chromosomes (Witten 1983). Our cell lines maintained normal chromosomal diploidy throughout the study. Chromosomes from all five species were used successfully in various cytogenetic analyses such as differential staining (DAPI, CA3, C-banding; Fig. 1b–d), C-banding (Fig. 1f) and fluorescent in situ hybridisation (Fig. 1e). We found no differences in banding, staining or FISH pattern when chromosomes were harvested from tail or toe clip fibroblasts when compared to those harvested from leukocytes or fibroblast cell lines originated form internal tissue culture. Of particular note are the chromosomes from *T. pinguicolla*, an endangered species and *A. norrisi* for which these preparations represent the first reported metaphase chromosomes and diploid chromosome numbers ($2n = 32$).

The protocol presented here is less labour intensive than traditional methods and could be easily adopted by any laboratory equipped for basic cell culture. Lizard toe and tail clips are routinely collected from wild animals in the course of ecological, evolutionary, taxonomic and conservation studies.

Establishing primary cell lines from external implants as we have demonstrated here, maximises the utility of samples that are often rare, expensive and difficult to collect, particularly in the case of endangered species. We have shown that primary cell

lines can be established from a very small ($\sim$3 mm) tissue explant without laborious trypsin or collagenase treatments. We are optimistic that this non-lethal technique can be used in other reptiles with appropriate modifications and, therefore, will provide research materials not only in cytogenetic studies but a wide variety of biological research.

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