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Comparative Efficiency of Different Sampling Techniques to Obtain DNA from Freshwater Turtles

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The advance of polymerase chain reaction (PCR) analysis has had an important impact on molecular ecology by allowing researchers to perform genetic analysis from small quantities of DNA. This modern technique, using non-lethal collection methods, is particularly useful when dealing with threatened species. Though blood sampling represents the predominant methodology for mammals (Sambrook et al., 1989), birds (Seutin et al., 1991), and reptiles (Haskell and Pokras, 1994), blood collection is invasive and can stress the animal and cause injuries. Alternative non-invasive sampling techniques would be beneficial for turtles and other small animals where blood sampling is difficult or potentially harmful.

Numerous tissue collection methods and extraction protocols have been designed to obtain DNA from old, dry, and degraded samples, such as fish scales (Tessier and Bernatchez, 1999), otoliths (Hutchinson et al., 1999), human fingernails (Ricci and Giovannuci Uzielli, 1996), mammal hairs (Goosens et al., 1999), and museum specimens (Ellegren, 1991; Thomas et al., 1990), among others. Re-

cently, Mockford et al. (1999) have shown that DNA could also be extracted from small fragments of carapace sampled from hatchling freshwater turtles. Their protocol may not apply to most adult specimens, however, after the shell becomes fully ossified. In this paper, we present an alternative non-invasive technique to obtain DNA from adult turtles, and then assess its relative efficiency in comparison with other sampling methods. The wood turtle (*Glyptemys insculpta*, formerly *Clemmys insculpta*), a threatened species in the USA and Canada, was used to validate this protocol.

Methods. — We lightly scraped the skin surface with a clean scalpel to remove superficial cell layers. We were able to sample large amounts of skin cells from the inguinal area, or from any other soft parts of the skin (e.g., limb surfaces and axillary areas). The cell scrapings were then placed in 95% ethanol to obtain a milky solution, which was used for DNA extraction. Alternatively, skin cells were collected by wiping off the scalpel blade in a paper envelope; the cells were further air-dried prior to genetic analyses. A few samples were also obtained by scraping off pieces of the shell scutes. Finally, blood samples (0.1 to 0.5 ml) were collected from a number of specimens by an antebrachial puncture, and kept in 1 ml of lysis buffer.

The same DNA extraction protocol was performed on all 39 samples: 18 from skin cells preserved in ethanol, 5 from air-dried skin cells, 12 from blood, and 4 from scute samples. Tissues preserved in ethanol were first centrifuged for 10 min before removing ethanol, whereas scute samples were cut in smaller pieces (1 to 2 mm) prior to DNA extraction. Samples were then individually placed in 500 µl extraction buffer (50mM Tris-HCl pH 8, 0.1 M EDTA, 1% SDS and 0.1 mg/ml Proteinase K) and incubated overnight at 37°C. A phenol-chloroform method was applied to purify the DNA, which was subsequently precipitated with 95% ethanol (for more details, see Sambrook et al., 1989). The amount of DNA extracted from the various samples was quantified with a spectrophotometer, at 260 nm. DNA quality for PCR amplifications was also tested with five microsatellite loci using specific primers designed for the bog turtle (*Glyptemys muhlenbergii*; Tim King, *pers. comm.*). In each case, 100 to 250 ng of DNA were used with a final concentration of 1x Buffer, 2 mM $MgCl_2$, 0.25 mM dNTP, 0.5 µM of each primer, and 0.5 U of Taq in a 10 µl volume reaction. Amplification products were analyzed on an ABI PRISM 310 automated sequencer.

Table 1. Results of DNA extractions and microsatellite amplifications for different types of tissues sampled from adult wood turtles. Five different loci with allele sizes ranging from 132 to 572 bps were used for the amplifications.

Tissue	No. Samples	Amount of DNA (ng)	No. Successful Amplifications	Success Rate
Blood	12	1,750-51,000	52	87%
Skin in ethanol	18	4,000-45,750	76	84%
Air-dried skin	5	3,550-22,000	19	76%
Scute	4	750-10,000	2	10%

Results and Discussion. — In most instances, comparable amounts of DNA were obtained from the different types of tissues (Table 1). Results of the microsatellite amplifications were also quite similar, except for the scute samples. In the latter case, the DNA extracted was not in good condition; only one successful amplification for two different loci was obtained for two specimens, even though we used as much as 480 ng of template DNA. With the exception of the scute samples, the results obtained from air-dried skin cells were not statistically different from those obtained from ethanol-preserved tissues or blood samples ($\chi^2 = 1.52$, $p = 0.47$).

Scraping superficial skin layers represents a good alternative technique to collect enough tissues for DNA extraction and genetic analyses. It is a non-destructive and non-invasive method that causes little stress compared to most other sampling techniques. This method can be used for adult specimens as well as for younger ones. It applies to different species of turtles and to a larger range of reptiles. For example, we have tested our procedure with five specimens of softshell turtles (*Apalone spinifera*). In all cases, the quality and amount of DNA obtained was adequate for subsequent genetic analyses. This method thus represents a simple and efficient alternative to blood sampling. It can be particularly useful for collecting tissues from threatened reptile species.

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Effect of Translocation on Egg Viability of the Giant Amazon River Turtle, *Podocnemis expansa*

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The giant Amazon river turtle (*Podocnemis expansa*) is the largest living South American freshwater turtle. Adult females have a flattened carapace up to 107 cm long (Ernst and Barbour, 1989). Its distribution includes the Orinoco and Essequibo to Amazon river drainages of Colombia, Venezuela, Guyana, eastern Ecuador, northeastern Peru, northern Brazil, and northern Bolivia (Iverson, 1992).

The eggs and meat of the species have been traditionally used by local people in the Brazilian Amazon, being socially important for the upper classes and economically important for the lower classes, which led the species to various levels of population decline in many parts of the Amazon (Pritchard and Trebbau, 1984; Alho, 1985). This decline stimulated the Brazilian government to establish in 1979 a ranching program of the species based on egg collection and captive raising of young for commercial purposes on a biologically sustainable basis (CENAQUA, 1989).

The species has a nesting behavior similar to that of some marine turtles with a markedly concentrated nesting period (Moll, 1979) which has been erroneously assumed to be related to low water levels (Alho and Pádua, 1982a; 1982b) and subsequent formation of sand beaches, known locally as *tabuleiros* (Alfinito, 1976). Average nesting female body mass can vary from 15.7 to 33.0 kg even in the same population and as many as some thousands may nest simultaneously (Ojasti, 1967) which is locally called an *arribada* in Spanish (Carr, 1967) or *arribação* in Portuguese (Alfinito, 1976). Females may possibly store sperm after copulation (van Tienhoven, 1983) and multiple paternity has been described (Valenzuela, 2000).

Although successfully used for caiman in Brazil (Verdade, 1985; Verdade et al., 1992) artificial incubation has been discarded as a management tool for the *P. expansa* ranching program because of the immense number of nests that would have to be housed (CENAQUA, 1989). However, a high rate of unhatched eggs have been reported in some *tabuleiros*, and the usual occurrence of unseasonal short-term flooding in the middle of the nesting period — known locally as *repiquete* (Pádua and Alho, 1984) — generally causes massive egg mortality (CENAQUA, 1985).

In order to minimize egg loss caused by this flooding without having to invest in artificial incubators, translocation of eggs has been proposed (Alho, 1985). However, its possible influence on egg viability has never been determined. This was the main goal of the present study.

Methods. — Eggs of *P. expansa* were collected from September to December 2000 in the following *tabuleiros* of Rio Araguaia: 04GO (Praia 04 GO: 13°21'57.6"S; 50°39'05.7"W) and 06 MT (13°23'32.3"S; 50°40'12.8"W), on the border between Goiás (GO) and Mato Grosso (MT) states, central Brazil. We tested four different translocation periods for the eggs, moving them at age 1, 14, 28, and 42 days since egg-laying.

We used eight nests per treatment (i.e., translocation period) with a total of 32 nests. The eggs were collected early in the morning (0700 to 0930 hrs) or late in the afternoon (1600 to 1830 hrs). Clutch size and the number of unviable eggs (damaged, decomposing, or no embryonic development) were recorded for each nest. Clutches were transported in separate sand-filled plastic boxes with the eggs placed in layers, reversed in relation to their original position in the nest (i.e., top layers placed in the bottom and vice-versa). The top of each egg was pencil-marked and eggs positions were maintained unaltered (i.e., eggs were not turned, so as to prevent egg mortality by respiratory or excretory dysfunction as suggested by Webb et al., 1987, for crocodile eggs).

All clutches were transported to a sand beach (18GO: 13°30'12.4"S; 50°44'12.4"W), a few kilometers from the *tabuleiros* of origin and less likely to be flooded. Careful transportation was by boat with the egg boxes eggs placed on a thick layer of foam and shaded by a layer of local soft weed (usually "*macela*", *Gnaphalium purpureum*) to prevent desiccation and overheating.

Artificial nests 50 cm deep and 25 cm wide were manually excavated one meter apart. The eggs were placed in the artificial nests in their original layer relationship. Local sand was gently spread over the eggs in order to fill up the nest without harming the eggs. Nests were individually identified by numbered stakes. A plastic fence (100 cm high, 15 cm buried in sand) was placed around the nests forming