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KARYOTYPIC EVOLUTION IN PLEUROCERID SNAILS. I. GENOMIC DNA ESTIMATED BY FLOW CYTOMETRY

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ABSTRACT

Total genomic DNA was measured in 16 species of North American pleurocerids, representing all six living genera. A constant value of 2.1 pg DNA/haploid genome was obtained, consistent with values from other mesogastropoods and other mollusks with similar chromosome number. The relationship between DNA content and evolutionary radiation is called into question.
Key words: Snails, freshwater, Pleuroceridae, cytogenetics, flow cytometry, DNA.

INTRODUCTION

Few groups of North American mollusks are as common, diverse, important, or poorly understood as the pleurocerid snails. They are the most conspicuous element of the macrobenthos in many rivers and streams, and as such have figured prominently in a number of ecological investigations (e.g. Elwood & Nelson, 1972; Sumner & McIntire, 1982; Hawkins & Furnish, 1987; Dillon & Davis, in review). Their occurrence in numerous, isolated populations, easily sampled year round, has made them ideal models for evolutionary research (Chambers, 1980, 1982; Dillon, 1984, 1988a, 1988b). Yet their systematics are so confused that the specific identity of the populations inhabiting much of the United States is problematic.

The first monographic treatment of the family was given by Tryon (1873). He catalogued about 500 nominal species, which he placed in nine genera. The currently accepted system of classification is due to a series of papers by Goodrich (e.g. 1940, 1942). Goodrich recognized somewhat over 100 species and subspecies, and his revision formed the basis of the classification by Burch & Tottenham (1980) and Burch (1982) that I use here. Burch recognized six living genera: *Io, Juga, Leptoxis* (including Goodrich's *Anculosa, Nitocris,* and *Eurycaelon*), *Lithasia, Pleurocera,* and *Elimia.* Burch resurrected "*Elimia*" on the strength of Pilsbry & Rhoads' (1896) type designation, failing to note that Pilsbry subsequently reversed himself (Walker, 1918:149). *Elimia* is a composite group, and thus I use the much more familiar name favored by Tryon and Goodrich, *Goniobasis.*

Very little distinguishes many of these genera. *Pleurocera* is distinguished from *Goniobasis* by a short "canal" on the anterior lip of the shell, a feature that is inconspicuous or absent in many species. Detailed comparisons of morphology, anatomy, life history, and ecology failed to find any other distinctions (Dazo, 1965). *Juga* is distinguished from *Goniobasis* by being from western North America, not eastern or central. It seems clear that the systematic relationships of pleurocerid snails need to be re-examined.

Analysis of karyotype has proven to be a powerful tool in evolutionary studies (White, 1973). But very little is known about the cytogenetics of any pleurocerid species. In a review of molluscan karyotypes, Patterson (1969) found two reports for North American pleurocerids: n = 18 for *Goniobasis laqueata* and n = 20 for *G. livescens.* Dillon (1982) reported n = 17 in *G. proxima.* The most thorough study to date has been made by Chambers (1982), who found n = 18 in Florida *Goniobasis* and described striking variation in arm length ratios. So the evidence available suggests that karyotypic variation does occur in the Pleuroceridae. In this series of papers, I will survey the six genera to see whether karyotype may be used to elucidate the systematics and evolution of this important family of freshwater snails.

Karyotypes are traditionally compared by constructing idiograms for each species, re-

producing the relative sizes and centromeric positions of the chromosomes. Idiograms are generally standardized to unit length, so that each species is assumed to have the same amount of genetic material. An increase in chromosome number is viewed as a centric fission. This is a reasonable assumption—many convincing examples of such "Robertsonian" events are known. But an increase in chromosome number could also represent additional genetic material. Additional chromosomes may be incorporated into a genome by coincident nondisjunction in the parents, or by large-scale gene duplication (Ohno, 1970). Thus it is of great value to determine the total genomic DNA content of each species to be karyotyped. In this first paper of the series, I estimate the genomic DNA content of a variety of pleurocerid snails using flow cytometry.

Flow cytometry is one of the most sensitive techniques available for quantifying cellular DNA. The technique has been used to discriminate between human chromosomes (Gray et al., 1975) and identify structural abnormalities in the chromosome compliments of cell lines (review by Arkesteijn et al., 1987). The technique has been thoroughly reviewed by Melamed et al. (1979), Van Dilla et al. (1985), and Shapiro (1988).

Briefly, tissues in an aqueous suspension are stained with a dye that intercalates into double-stranded nucleic acid. I used propidium iodide, after treatment with ribonuclease to eliminate double-stranded RNA. Then the suspension is channeled at high speed through a narrow aperture, using mechanics similar to those of the familiar Coulter counter. Each individual particle passes through a laser, which excites a red-fluorescent emission proportional to its DNA content. The degree to which the laser beam is scattered by each particle provides an estimate of the particle's size. The emissions of the individual particles are captured by photosensors and displayed in a scatter plot, which enables the operator to distinguish individual, whole cells from debris and clumped cells. Then the red fluorescence of the whole-cell fraction is plotted in a histogram, with fluorescence measured in arbitrary units called "channel numbers." Since the relationship between channel number and DNA content is effectively linear, a flow cytometer calibrated with known samples can be used to estimate the DNA content of an unknown.

The contribution of mitochondrial DNA to total red fluorescence has generally been found to be negligible (Melamed et al., 1979). Correction for any background mtDNA levels can be made by using a single tissue type for both the unknowns and the calibration standards.

METHODS

The following populations were sampled:

Goniobasis acutocarinata (Lea)—Small creek flowing into the Powell River at Virginia Highway 662 bridge, 0.5 km E of Stickeys, Lee County, Virginia. Goodrich (1940) synonymized this species under *G. clavaeformis* (Lea).

Goniobasis alabamensis (Lea)—Coosa River at tailwater of Mitchell Dam, 20 km E of Clanton, Chilton County, Alabama.

Goniobasis catenaria dislocata (Reeve)—"Intermittent" tributary of Big Poplar Creek at South Carolina Highway 6 bridge, 3 km SE of Elloree, Orangeburg County, South Carolina.

Goniobasis floridensis (Reeve)—Blue Spring at Florida Highway 6, Madison County, Florida. Site 8 of Chambers (1980).

Goniobasis livescens (Menke)—Portage Creek at Toma Road bridge, 5 km S of Pinckney, Washtenaw/Livingston County line, Michigan. Station 2 of Dazo (1965).

Goniobasis proxima (Say)—Mitchell River at North Carolina Highway 1330 bridge, 2.8 km N of Mountain Park, Surrey County, North Carolina. Site MTCH of Dillon (1982, 1984).

Goniobasis simplex (Say)—same site as *G. acutocarinata.*

Io fluvialis (Say)—Powell River by small road just S of Virginia line, Hancock County, Tennessee.

Juga hemphilli (Henderson)—Oak Creek 11 km W of Corvallis, Benton County, Oregon.

Leptoxis (Mudalia) carinata (Brug.)—Pratts Run at U.S. Highway 340 bridge, Waynesboro, Augusta County, Virginia.

Leptoxis praerosa (Say—same site as *Io fluvialis.*

Lithasia duttoniana (Lea)—Duck River at Tennessee Highway 11 bridge, 10 km N of Farmington, Marshall County, Tennessee.

Lithasia verrucosa (Raf.)—French Broad River at Cement Shoals, 1 km downstream from Kimberlin Heights, Knox County, Tennessee.

Pleurocera acuta Raf.—Dazo's (1965) station 2, same as *G. livescens.*

Pleurocera canaliculatum (Say)—Elk River

FIG. 1. From left, *Lithasia duttoniana, Goniobasis catenaria dislocata, G. alabamensis, G. acutocarinata, Juga hemphilli.*

at bridge 8 km W of Fayetteville, Lincoln County, Tennessee.

Pleurocera unciale (Reeve)—same site as *Io fluvialis.*

The shell morphology of many of these species is quite variable, as are the species concepts of many prior workers in pleurocerid taxonomy. Typical shells from several of the less common taxa are shown in Fig. 1. Voucher specimens for all populations are deposited in the Academy of Natural Sciences of Philadelphia.

Techniques for sample preparation were based on Allen (1983), Buzzi (1989), and standard clinical methods. Foot muscle was excised from living snails and ground, with powdered glass, in a clear polystyrene tube with 600 μl of phosphate buffered saline. This buffer was modified from Allen (1983): NaCl 8.0 g/l, KCl 0.20 g/l, $MgCl_2$ 0.10 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.20 g/l. Drops of cold absolute ethanol were added, while vortexing, to bring the final ethanol concentration up to 70%. Samples fixed in this manner were held at least overnight at 3°C, and sometimes as long as two weeks.

An RNAse solution was prepared by dissolving 50 mg ribonuclease A (Sigma type III-A) in 50 ml 1.12% trisodium citrate and heating at 80°C for 10 minutes. The solution was frozen in 2 ml aliquots. On the morning of flow cytometric analysis, fixed tissue samples were centrifuged, aspirated, and resuspended in a several ml of a solution containing 47 ml phosphate buffered saline, 1 ml 1.2% (v/v) Nonidet P-40, and one aliquot of RNAse solution. Since the lot of ribonuclease III-A that I received had 105 units of activity per ml, the final RNAse activity in phosphate buffered saline was approximately 4 units/ml. Tubes were incubated at room temperature for 30–60 minutes.

Each sample was vortexed, drawn into a 1 ml tuberculin syringe, and forced through a 52 μm Nytex screen. Then 20 μl of a 0.10% (w/v) propidium iodide solution was added per ml of tissue suspension, and incubated at room temperature for 30–60 minutes prior to analysis on an Ortho Spectrum III flow cytometer.

In collaboration with W. Buzzi, a calibration curve was constructed using human leukocytes and tissue samples from four mollusk species of known genomic content. We analysed four *Crassostrea virginica* (Gmelin), five *Mercenaria mercenaria* (L.), and two *Ilyanassa obsoleta* (Say), all collected from the Charleston, South Carolina, area. We obtained four *Mytilus edulis* L. from Milford, Connecticut. The total genomic DNA of *C. virginica* is given by Swanson et al. (1981:134), and values for the remaining mollusks are from Hinegardner (1974). Three individuals of *G. catenaria dislocata* were included as unknowns.

Goniobasis catenaria dislocata served as the standard in all subsequent analyses. Several fresh *G. catenaria* were analysed first, followed by four to six individuals of a second pleurocerid species. The peak red fluorescence for each sample was noted, as well as the concentration of countable cells. Aliquots from samples of the two species were combined into a third tube such that cell concentrations were equalized. The combined sample was then re-analysed and the resulting histogram of red fluorescence inspected for

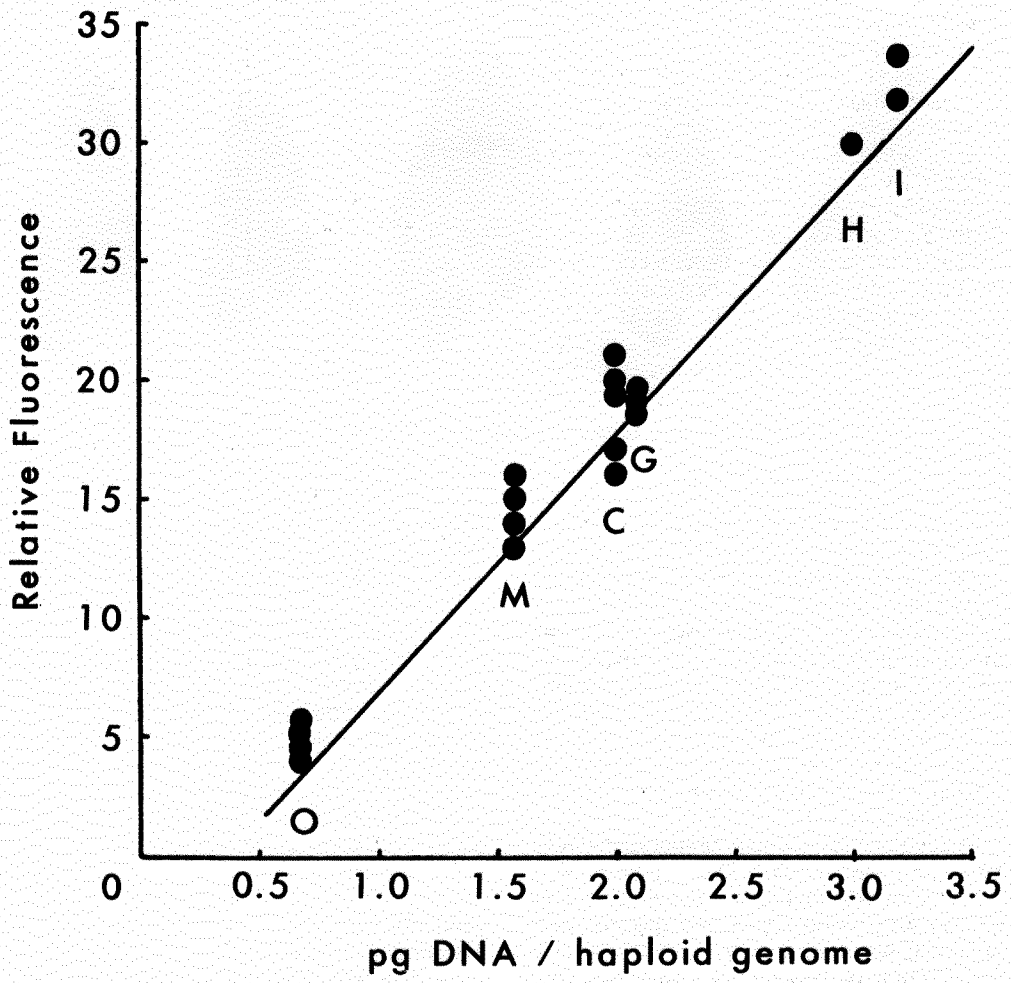

FIG. 2. Calibration curve. O—*Crassostrea,* M—*Mytilus,* C—*Mercenaria,* G—*Goniobasis catenaria dislocata,* H—human, I—*Ilyanassa.*

evidence that the two peaks were non-overlapping.

RESULTS

The calibration curve is shown in Fig. 2. An excellent fit to the linear hypothesis $y = 10.8x - 3.76$ was obtained, with $r^2 = 0.98$. So given a mean relative fluorescence of 17.3, I estimated that *G. catenaria dislocata* has 2.1 pg DNA/haploid genome.

A typical comparison between *G. catenaria dislocata* and an unknown (*G. proxima* in this case) is shown in Fig. 3. This particular sample of *G. catenaria* tissue came from a snail collected the previous day, and shows two peaks—a strong gap 1 peak and a lower gap 2/mitosis peak with twice the fluorescence. Only snails freshly collected in warm weather generally showed a gap 2 peak. Even if temperature and photoperiod were controlled and the snails fed commercial fish food *ad lib,* gap 2 peaks generally disappeared after only a day or so in captivity, as shown in the *G. proxima* sample. In fact, it is evident that DNA synthesis has already been discontinued in the *G. catenaria* individual analysed, since no S-phase cells, with DNA contents intermediate between G1 and G2, are apparent in Figure 3. So although the snails in my aquaria always appeared healthy, cell division in foot

muscle tissue was apparently disrupted almost immediately.

The rapid loss of cells at gap 2 and mitosis in captive snails did not affect the accuracy of sample comparisons. The much stronger, sharper gap 1 peaks were used as the basis for comparison in all cases.

No difference was detected between the peak red fluorescence of *G. catenaria dislocata* and that observed in any other species of pleurocerid examined. Figure 3 shows that an equal mixture of *G. catenaria* cells and *G. proxima* cells shows no evidence of two gap 1 peaks. This result was obtained in all comparisons.

DISCUSSION

It would appear that all 16 pleurocerid species in my sample, representing six genera, have a uniform genomic DNA content of 2.1 pg DNA/haploid genome. Hinegardner (1974) found that seven species of mesogastropods range from 0.67–2.4 pg DNA/haploid genome. A vermetid was the only cerithiacean examined, with 1.5 pg DNA/haploid genome. So the value I have obtained for pleurocerids is consistent.

From a broad comparison of gastropod orders, Hinegardner suggested that high amounts of DNA appear to be associated with evolutionary radiation. But in spite of their rather average-sized genome, the Pleuroceridae have radiated extensively. Hinegardner's generalization may not hold for freshwater groups, where dispersal is generally much more restricted and the potential for differentiation greater.

Across the five kingdoms, there is a general relationship between genome size and degree of organismal complexity or "evolutionary advancement" (Hinegardner, 1976). The "C-value paradox" arose when it was noted that some organisms, such as some flowering plants and amphibians, have amounts of DNA ("C-values") much greater than more advanced eukaryotes. But the pleurocerid genome size is rather typical for mollusks, and for invertebrates in general.

Hinegardner (1974) reported a correlation between chromosome number and DNA content in gastropods significant at the 0.01 level. Extrapolating from his graph, a chromosome number of n = 13 to 18 would be predicted from the DNA content of North American pleurocerids. This is consistent with the limited information available on pleurocerid karyotypes. Ongoing studies will more thoroughly address the degree to which uniformity in genomic DNA content reflects karyotypic conservation in this family. Any variation in chromosome number among pleurocerids can be

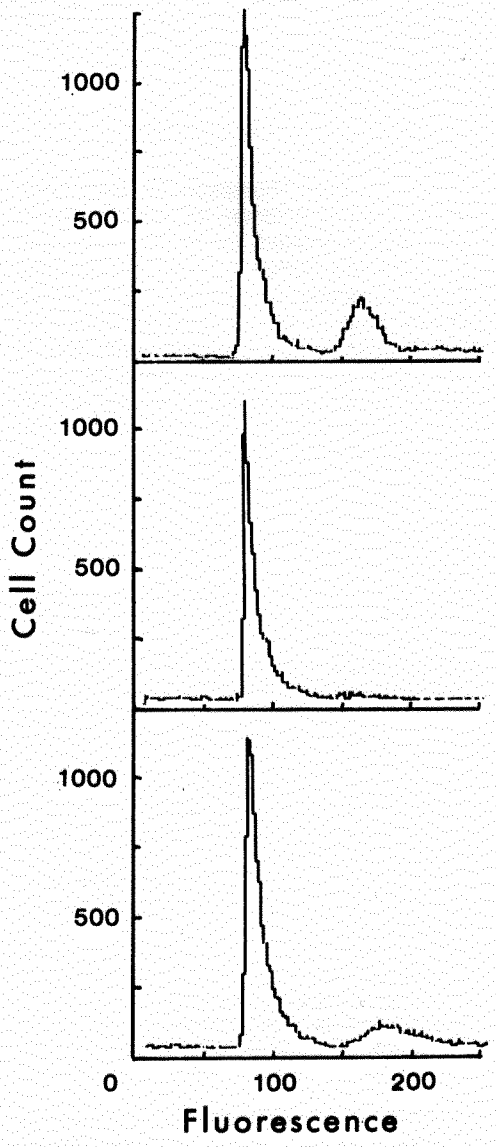

FIG. 3. Example comparison of unknown and standard. Top—fresh *G. catenaria dislocata* standard, showing gap 1 and gap 2 peaks. Middle—the unknown (*G. proxima*), showing gap 1 peak only. Bottom—equal mixture of standard and unknown, demonstrating complete overlap of gap 1 peaks.

viewed with some confidence as originating in Robertsonian fusion or fission.

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