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PATTERNS OF GENOME SIZE EVOLUTION IN TETRAODONTIFORM FISHES

ELIZABETH L. BRAINERD,[1] SANDRA S. SLUTZ,[2] EDWARD K. HALL,[3] AND RANDALL W. PHILLIS
Department of Biology, University of Massachusetts, 611 North Pleasant Street, Amherst, Massachusetts 01003-9297
[1]*E-mail: brainerd@bio.umass.edu*

Abstract.—We used flow cytometry to measure genome size in 15 species from seven families and subfamilies of tetraodontiform fishes. Previous studies have found that smooth pufferfishes (Tetraodontidae) have the smallest genome of any vertebrate measured to date (0.7–1.0 picograms diploid). We found that spiny pufferfishes (Diodontidae, sister group to the smooth puffers) possess a genome that is about two times larger (1.6–1.8 pg). *Mola mola*, a member of the sister group to Diodontidae and Tetraodontidae, also has a relatively large genome (1.7 pg). Parsimony analysis of this pattern indicates that the plesiomorphic condition for Molidae (Diodontidae, Tetraodontidae) is a genome size of 1.6–1.8 pg, and that tiny genome size is a derived character unique to smooth puffers. However, an alternative explanation is that the ancestor of Tetraodontidae acquired a heritable tendency toward decreasing genome size, such as a new or modified deletion mechanism, and genome size in all of the tetraodontid lineages has been decreasing in parallel since the split from Diodontidae. Small genome size (1.1–1.3 pg) also appears to have evolved independently in some members of Balistoidea (triggerfishes and filefishes) within Tetraodontiformes.

Key words.—Diodontidae, genome size, Molidae, nucleotype, pufferfish, Tetraodontidae, Tetraodontiformes.

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Diploid genome size, measured in picograms (1 pg = 10^{-12} g) of DNA per cell, varies widely among vertebrates. The largest vertebrate genome size measured to date is 284 pg in an African lungfish (*Protopterus aethiopicus*; Pedersen 1971). The smallest vertebrate genome size measured to date is 0.70 pg in a smooth pufferfish (*Tetraodon fluviatilis*; Lamatsch et al. 2000). This large interspecific variation in genome size cannot be explained by changes in the amount of coding DNA from one species to another, nor do there appear to be consistent patterns in the evolution of smaller or larger genomes within vertebrates (Cavalier-Smith 1985).

Genome size has, however, been found to correlate with several organismal traits. In salamanders, genome size is correlated with cell size and is inversely correlated with cell number, limb regeneration rate, and embryonic developmental rate (Olmo and Morescalchi 1975, 1978; Sessions and Larson 1987; Jockusch 1997; Roth et al. 1994, 1997). In mammals and birds, genome size is correlated with cell size and is inversely correlated with metabolic rate (Vinogradov 1995, 1997). These correlations are thought to result from effects of the size of the nucleus on cell size and cell division rates, and thus are called nucleotypic effects (Bennett 1971).

Theories to explain the observed patterns of genome size variation tend to fall into two broad categories: those in which natural selection is viewed as the primary mechanism controlling genome size and those in which variation in genome size is thought to be essentially neutral to natural selection, with genome size instead dependent on stochastic processes and historical accident (Cavalier-Smith 1985; Wachtel and Tiersch 1993; Petrov 2001). However, these two points of view do share a common basis: they agree that genome size results from a balance between (1) genome-level mechanisms which tend to increase genome size, such as duplication, transposable elements, and polyploidy; and (2) genome-level mechanisms which tend to decrease genome size, such as spontaneous deletions and DNA repair mechanisms (Petrov 2001).

In teleost fishes, genome size in a phylogenetically diverse sample of 275 species has been found to vary from less than 1 pg to almost 9 pg (Fig. 1, replotted from data in Hinegardner and Rosen 1972). The distribution of genome sizes clusters tightly (relative to the full range of genome sizes in vertebrates) around a modal genome size of 2.0 pg and is skewed to the right. The smallest vertebrate genome sizes measured to date are found in the smooth pufferfishes (Tetraodontidae), which have genome sizes in the range of 0.7–1.0 pg (Hinegardner and Rosen 1972; Brenner et al. 1993; Lamatsch et al. 2000). The next largest genomes are 20–30% larger (1.2–1.3 pg), and are found in many members of Gasterosteiformes (sticklebacks, seahorses, and pipefishes), in several members of Anabantoidei (bettas and gouramis) and in a few members of Pleuronectiformes (flounders and soles) (Hinegardner 1968; Hinegardner and Rosen 1972).

The highly reduced genome size in Tetraodontidae suggests that puffers and their relatives may be a good model system in which to study the evolution of small genome size in vertebrates. Tetraodontiform fishes may also be a good model system because the genome of a pufferfish, *Fugu rubripes*, has been proposed as a model genome for sequencing and gene identification (Brenner et al. 1993), and a plan to sequence the full genome of *F. rubripes* has been announced (http://fugu.hgmp.mrc.ac.uk). Pufferfish genomics may provide important information for understanding the evolution of genome size in these fishes.

The purpose of the present study is to determine the patterns of genome size evolution in Tetraodontiformes, an order that includes smooth puffers (Tetraodontidae), spiny puffers (Diodontidae), ocean sunfishes (Molidae), boxfishes and

[2] Present address: Department of Genetics, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305-5120.

[3] Present address: Department of Ecology, Evolution and Behavior, University of Minnesota, 1987 Upper Buford Circle, St. Paul, Minnesota 55108.

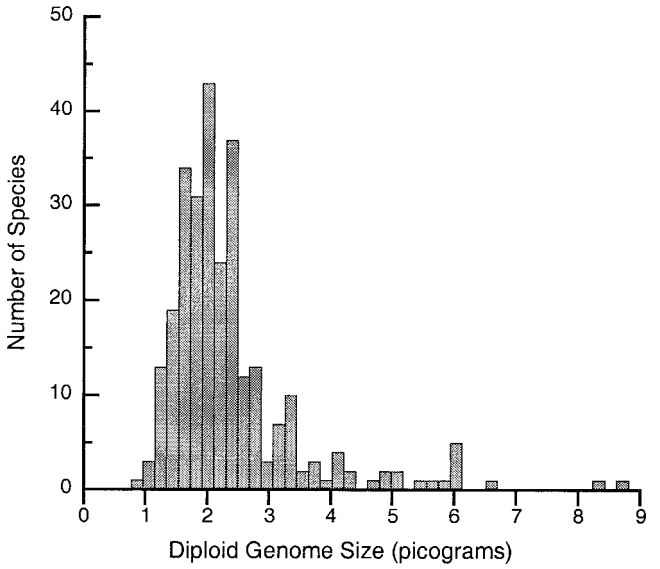

FIG. 1. Distribution of diploid genome sizes in 275 species of teleost fishes (replotted from data in Hinegardner and Rosen 1972).

trunkfishes (Ostraciidae), triggerfishes (Balistidae), filefishes (Monacanthidae), and spikefishes and triplespines (Triacanthoidea). Tetraodontiform fishes are unusual in that they rely heavily on median and paired fin swimming (rather than body-caudal fin swimming), have elaborate mechanical defenses, and have fewer skeletal elements than most other fishes (Tyler 1980; Brainerd 1994; Wainwright et al. 1995; Brainerd and Patek 1998). Low vertebral number (22 or fewer vertebrae) and absence of pleural ribs are synapomorphies for Tetraodontiformes. In addition, tetraodontids, diodontids, and molids lack pelvic fins, spines, and girdles, and molids have the fewest vertebrae of any fish (16 in *Mola mola*) and completely lack all caudal bones (no hypurals, epurals, or uroneurals).

Hinegardner and Rosen (1972) proposed that small genome size in some groups of fishes might be related to the loss of anatomical parts relative to their ancestral conditions. If a lineage is experiencing strong, genome-level pressure toward genome size reduction, then it is possible that some coding regions might be lost. This would only be true if pressure for the loss of genetic material is stronger than natural selection against loss of the coding region. One goal of the present study was to test this hypothesis in tetraodontiform fishes. If loss of bony elements is correlated with loss of DNA, then we predict that: (1) molids should have the smallest genome size (because they have the most reduced skeleton); (2) tetraodontids and diodontids should have the second smallest genome size; and (3) the genomes of the rest of the tetraodontiforms should be larger than those of ocean sunfishes and puffers but smaller than the genomes of fishes with more skeletal elements. We have tested these predictions by measuring genome size in 15 species of tetraodontiform fishes representing seven families or subfamilies, and mapping these data onto a family-level phylogeny to reconstruct the pattern of genome size evolution in tetraodontiform fishes.

MATERIALS AND METHODS

We used flow cytometry to measure genome size (diploid DNA content) of red blood cells from a total of 45 individuals belonging to 15 species and seven families and subfamilies within Tetraodontiformes. Preserved museum specimens could not be used for this study because fresh, unclotted blood was required for flow cytometry (blood may be frozen for storage but must be collected fresh). The need to obtain fresh blood prevented us from measuring genome size in the more rare tetraodontiforms (Triacanthoidea and *Triodon*), and limited our sample sizes for number of individuals within each species, number of species within genera, and number of genera within families. Fresh blood from *Mola mola* was particularly difficult to obtain, but eventually we were able to obtain one fresh specimen (estimated mass 100 kg) from a fisherman in the Atlantic tuna harpoon fishery off the northern coast of Massachusetts. We were not able to obtain fresh blood samples from the relatively rare and mostly deep water triacanthoids, nor from the monotypic family Triodontidae, which is known from only a few museum specimens. For comparison with our sampling, the numbers of genera and species recognized within each group are as follows (Nelson 1994): Triacanthoidea (13 genera, 22 species); Balistidae (11 genera, 40 species); Monacanthidae (31 genera, 95 species); Ostraciidae (14 genera, 33 species); Triodontidae (1 genus, 1 species); Tetraodontidae (9 genera, 121 species); Diodontidae (6 genera, 19 species); and Molidae (3 genera, 3 species).

Specimens used for genome size analysis came from four sources: *Cantherhines pullus, Diodon holocanthus, Diodon hystrix, Lactophrys bicaudalis, Xanthichthys ringens, Monacanthus tuckeri,* two *Canthigaster rostrata,* and one *Melichthys niger* were collected off the northern coast of Jamaica; one specimen of *Mola mola* (estimated mass 100 kg) was collected by harpoon fishing off the northern coast of Massachusetts; *Spheroides nephelus* and *Monacanthus hispidus* were purchased from Gulf Specimen Marine Lab (Panacea, FL); *Chilomycterus schoepfi, Lactophrys quadricornis, Monacanthus ciliatus, Tetraodon fluviatilis,* one *Canthigaster rostrata,* and one *Melicthys niger* were acquired through the aquarium trade.

Standard flow cytometric methods for measuring genome size were used (e.g., Vindelov et al. 1983; Johnson et al. 1987; Lockwood and Bickham 1991). Briefly, blood from large specimens, such as *Mola mola,* was collected from the sinus venosus of the heart. Small specimens were killed by an overdose of tricaine methanesulfonate and immediately bled from the caudal artery into 1 ml of acid citrate dextrose anticoagulant solution (15% citric acid, 40% sodium citrate, 45% dextrose). Red blood cell concentration was diluted to 10^6 cells per ml and filtered through a 35-μ nylon mesh. Whole chicken blood, previously frozen in DMSO as described by Vindelov et al. (1983), was thawed and added to the fish sample as an internal standard in a 3:1 ratio of fish to chicken cells. Cells were stained with a propidium iodide (PI) solution (Vindelov et al. 1983) at a 1:1 volume ratio of PI solution to cell sample. The cell solutions were incubated in darkness for 30 min, and then the fluorescence of 10,000–

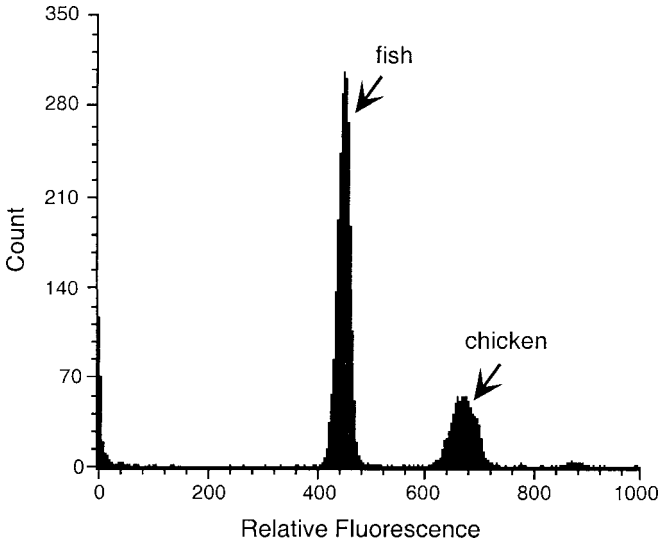

FIG. 2. Relative fluorescence histogram for propidium iodide stained erythrocytes from an ocean sunfish, *Mola mola,* (left peak) and a chicken (right peak) as measured by flow cytometry (10,000 counts recorded). Chicken blood was added to each sample as an internal standard for calculation of genome size in picograms (genome size of the fish is equal to ratio of fish to chicken fluorescence multiplied by the known chicken genome size, 2.54 pg). The fish peak is approximately three times higher than the chicken peak because the relative concentration of fish to chicken cells in the sample was set at 3:1.

20,000 cells per sample was measured on a Becton Dickinson (San Jose, CA) FACScan.

Diploid genome size in picograms was calculated using the known genome size of chicken blood cells, which served as an internal standard (chicken 2 n = 2.54 pg; Rasch et al. 1971). The genome size of each fish sample was calculated by multiplying the fish to chicken fluorescence ratio by the known value of the chicken genome size. The fluorescence ratio was derived from the median values of the chicken and fish peaks in each sample's fluorescence histogram. Chicken and fish peaks were identified by height (Fig. 2); the fish peak was always higher because the samples contained a greater concentration of fish cells (3:1 ratio of fish to chicken cells).

Patterns of genome size evolution were explored by mapping the range of genome sizes available for each family or subfamily onto a phylogenetic hypothesis of tetraodontiform relationships (Winterbottom 1974). Because relationships of genera within the families of tetraodontiforms are poorly known, we have chosen to map the full range of genome sizes for each family (combined range of all available values for species within each family including intraspecific variability and literature values) rather than attempting to calculate phylogenetically corrected means. Mapping the full range of genome sizes for each family is a conservative approach to mapping these data onto a cladogram, and patterns emerging from this relatively low resolution approach are likely to reflect strong signals in the data.

The tetraodontiform phylogeny we use for our analysis is based on myological characters (Winterbottom 1974) and is congruent with relationships based on osteological characters (Tyler 1980; Tyler and Sorbini 1996). After Nelson (1994), we recognize nine families of tetraodontiforms: Triacanthodidae, Triacanthidae, Monacanthidae, Balistidae, Ostraciidae, Triodontidae, Tetraodontidae, Diodontidae, and Molidae. We recognize two subfamilies of tetraodontids, Tetraodontinae and Canthigasterinae. We use the name Triacanthoidea to indicate the clade containing Triacanthodidae and Triacanthidae and the name Balistoidea to indicate the clade containing Monacanthidae and Balistidae.

TABLE 1. Diploid genome size measurements in picograms per cell.

Species	n	Mean	Range	Literature values[1]
Canthigasterinae				
Canthigaster rostrata	3	0.90	0.86–0.94	—
Tetraodontinae				
Fugu rubripes	—	—	—	0.83[2]
Spheroides maculatus	—	—	—	1.00
Spheroides nephelus	2	0.91	0.91–0.91	1.00
Tetraodon palembangensis	—	—	—	0.96
Tetraodon fluviatilis	5	0.80	0.78–0.83	0.78 0.70[3]
Diodontidae				
Chilomycterus schoepfi	6	1.60	1.56–1.63	1.80
Diodon holocanthus	2	1.58	1.55–1.60	—
Diodon hystrix	1	1.63	—	—
Molidae				
Mola mola	1	1.70	—	—
Ostraciidae				
Lactophrys bicaudalis	1	1.99	—	—
Lactophrys quadricornis	6	2.22	2.20–2.26	—
Lactophrys trigonis	—	—	—	1.70
Lactophrys triqueter	—	—	—	2.20
Monacanthidae				
Cantherhines pullus	3	1.17	1.17–1.18	—
Monacanthus ciliatus	3	1.17	1.16–1.18	—
Monacanthus hispidus	5	1.25	1.21–1.27	—
Monacanthus tuckeri	4	1.17	1.14–1.21	—
Balistidae				
Aluterus schoepfi	—	—	—	1.28
Balistes sp.	—	—	—	1.44
Melichthys niger	2	1.42	1.36–1.48	—
Stephanolepis hispidus	—	—	—	1.36
Xanthichthys ringens	2	1.49	1.48–1.49	—

[1] Literature values from Hinegardner and Rosen 1972 unless otherwise specified.
[2] From Brenner et al. 1993.
[3] From Lamatsch et al. 2000.

RESULTS

Genome size (diploid DNA content per cell) measurements from 15 species belonging to seven families and subfamilies of Tetraodontiformes are presented in Table 1. For comparison with our measurements, Table 1 also includes genome sizes available from the literature (converted to diploid DNA content). Exceptionally small genome sizes were found in all sampled members of Tetraodontidae (Table 1 and Fig. 3). One species in the monogeneric subfamily Canthigasterinae was found to have a mean genome size of 0.90 pg. Our measurements of two species within Tetraodontinae yielded a range of 0.78–0.91 pg for members of this subfamily, which

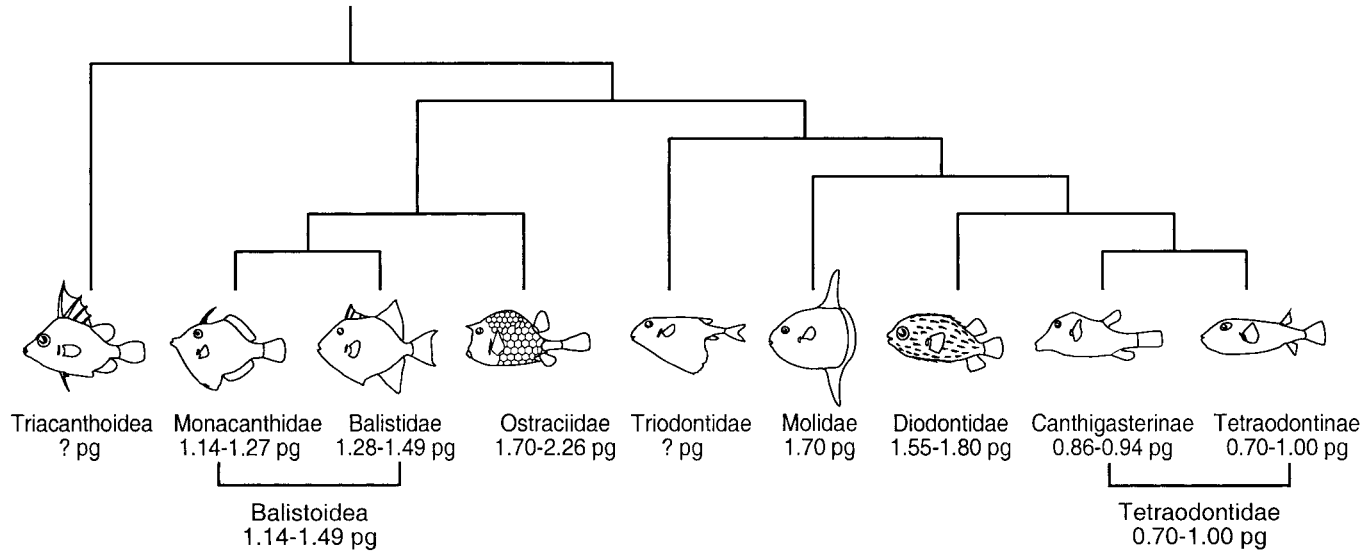

FIG. 3. Range of genome sizes for sampled members of seven tetraodontiform clades mapped onto a phylogenetic hypothesis of familial relationships (phylogeny based on morphological characters, Winterbottom 1974). Values in the range include both new measurements reported in this paper and values from the literature (Table 1).

is contained within the range of 0.70–1.00 pg from literature values (Hinegardner and Rosen 1972; Lamatsch et al. 2000).

Genome size in members of Diodontidae (sister group to Tetraodontidae) was found to be considerably larger than the genome size of tetraodontids (1.55–1.63 pg in our sampled diodontids). The ocean sunfishes (Molidae) are the sister group to the puffers (Tetraodontidae and Diodontidae). The genome size of one specimen of *Mola mola* was found to be 1.70 pg, which is similar to that of the spiny puffers (Fig. 3).

Genome size in members of other tetraodontiform families was found to vary widely, from 1.14 to 2.26 pg (Table 1 and Fig. 3). Members of Monacanthidae and Balistidae were found to have fairly small genome sizes, whereas members of genus *Lactophrys* (Ostraciidae) had the largest genome size of all tetraodontiforms sampled (2.20–2.26 pg).

DISCUSSION

Patterns of Genome Size Evolution in Tetraodontiform Fishes

Our measurements confirm that members of Tetraodontidae (smooth puffers) possess the smallest vertebrate genome measured to date (Table 1 and Fig. 3). However, this tiny genome size is not characteristic of all tetraodontiform fishes, nor even of all puffers. Members of Diodontidae (spiny puffers) possess a genome that is nearly two times larger (1.6–1.8 pg). *Mola mola,* a member of the sister group to Tetraodontidae and Diodontidae, also has a relatively large genome (1.7 pg). Although the genome size of diodontids is almost exactly twice that of the tetraodontids, the difference in genome size between spiny and smooth puffers does not appear to result from differences in ploidy. Chromosome number ranges from 34 to 44 in smooth puffers, and the one spiny puffer karyotyped to date, *Diodon bleekeri,* was found to have 46 chromosomes (Arai and Nagaiwa 1976; Choudhury et al. 1979).

Parsimony analysis of the phylogenetic pattern of genome size suggests that the plesiomorphic condition for Molidae (Tetraodontidae, Diodontidae) is a genome size of 1.6–1.8 pg, and the tiny genome of Tetraodontidae is a derived character unique to the smooth puffers (Fig. 3). If we accept this parsimony analysis, then we conclude that small genome size evolved sometime after the split between Tetraodontidae and Diodontidae, and genome size in the Tetraodontidae has remained low ever since. Paleontological evidence indicates that the families of tetraodontiform fishes diverged from each other between approximately 90 and 50 million years ago (Tyler and Sorbini 1996). Extinct species that can be referred to most of the extant families are present in fossils from the Eocene, and fossils that are clearly tetraodontiforms but not referable to any extant family are known from the upper Cretaceous (Tyler and Sorbini 1996). This analysis suggests that genome size in Tetraodontidae has been static for at least 50 million years.

An alternative explanation for the observed patterns of genome size yields a very different interpretation of the evolution of genome size in this group. Studies of genome size evolution in *Drosophila* and crickets indicate that it is possible for lineages to acquire heritable deletion mechanisms that drive down genome size over time (Petrov et al. 1996, 2000; Petrov 2001). An alternative explanation for the small genome size in all tetraodontids is that the ancestor of Tetraodontidae had relatively large genome size, but acquired the tendency to lose DNA over time. In this scenario, all of the smooth puffers have been gradually losing DNA since they diverged from the spiny puffers, and the tiny genomes we see today are the result of parallel, directional evolution in genome size. In this alternative explanation, genome size has not been static for the past 50 million years, but rather has been gradually decreasing in all of the lineages within Tetraodontidae during that time.

This alternative scenario highlights a difficulty with parsimony based, comparative analyses of genome size evolu-

tion. If future studies find that heritable tendencies toward increasing or decreasing genome size are common, then it will not be possible to use only the pattern of genome size and parsimony analysis to reconstruct ancestral genome sizes. It may be possible to determine whether an entire clade inherited small or large genome size from an ancestor or whether genome size has been evolving in parallel within the clade by comparing the sequences of pseudogenes and other neutrally evolving parts of the genome, but a methodology has not yet been developed for this.

Within the Tetraodontiformes, small genome size has also evolved independently in Balistoidea (Fig. 3). Mean genome size of the two monacanthid genera sampled is 1.2 pg, which is 30% larger than the genome size of the tetraodontids, but is still among the smallest vertebrate genomes measured to date. The only other fishes with genome sizes equal to or less than 1.3 pg are most members of Gasterosteidae (sticklebacks, seahorses and pipefishes, 1.2–1.3 pg), a few members of Pleuronectiformes (flounders and soles, 1.3 pg), and several members of Anabantoidei (bettas and gouramis, 1.2–1.3 pg) (all values from Hinegardner and Rosen 1972). Genome sizes between 1.2 and 1.3 pg are thus rare, but have evolved more than once in fishes, whereas a genome size below 1.0 pg appears to have evolved only in Tetraodontidae.

Small Genome Size and Loss of Skeletal Elements

Our results do not support the hypothesis that small genome size is correlated with loss of anatomical parts (Hinegardner and Rosen 1972). *Mola mola* has the most reduced skeleton among the tetraodontiforms we sampled, with pleural ribs, pelvis, and caudal bones absent and only 16 vertebrae present. *Mola mola* did not, however, have the smallest genome (Fig. 3). In addition, both families of puffers (Tetraodontidae and Diodontidae) have equally reduced skeletons and similar overall morphology, but diodontids have nearly two times more DNA than tetraodontids. These results do not indicate that there is strong correlation between loss of skeletal elements and reduction in genome size in tetraodontiform fishes.

Organismal Traits Correlated with Genome Size

In other groups of vertebrates, genome size has been found to be correlated with cell volume, and to be inversely correlated with cell number, regeneration rates, developmental rates, and metabolic rates (Olmo and Morescalchi 1975, 1978; Sessions and Larson 1987; Vinogradov 1995, 1997; Jockusch 1997; Roth et al. 1994; 1997). These nucleotypic effects are thought to be caused by the size of the nucleus, which affects cell volume, surface to volume ratio of cells, and the rate of cell cycling. At this time, cell size and rates of regeneration, development, and metabolism are unknown for tetraodontids, diodontids, and molids. However, the presence of small genome size in tetraodontids and larger genome size in the closest outgroups suggests that this would be a good group in which to study nucleotypic effects of small genome size.

Mechanisms of Genome Size Evolution

Genome size is thought to evolve as a result of shifts in the balance between (1) genome-level processes which produce insertions and cause genome size to increase, and (2) genome-level processes which produce deletions and cause genome size to decrease (Petrov et al. 2000; Petrov 2001). However, this statement is deceptively simple: major questions remain as to the exact nature of these processes and whether the balance between them in any particular lineage results primarily from the action of natural selection or from stochastic changes in the processes themselves.

In tetraodontiforms, the small genome size of tetraodontids appears to have evolved independently of the small genome size of monacanthids (Fig. 3). The reduction of genome size twice within Tetraodontiformes suggests that there may be a genome-level mechanism in this group that promotes genome size reduction. Studies of genome size in *Drosophila* have found a high rate of DNA deletions, which counteracts insertions and may help maintain a small genome size (Petrov et al. 1996, 2000). Bats may also have a genome-level mechanism for maintaining low genome size, but no specific mechanism has been proposed (Baker et al. 1992).

Tetraodontiform Fishes as a Model System for Studies of Genome Size Evolution

We propose that tetraodontiform fishes are a good model system in which to study the evolutionary mechanisms of genome size reduction. A weakness of tetraodontiforms as a model system is the difficulty of keeping and breeding live specimens, which makes studies of developmental rates and gene expression during development impractical at this time (although brackish water puffers in the genus *Tetraodon* are easier to keep than the marine puffers). This weakness is balanced by four strengths: (1) the smallest genome of any vertebrate is found in Tetraodontidae; (2) the two closest outgroups to tetraodontids have genome sizes that are almost two times larger, thereby facilitating comparisons between closely related lineages having small and large genomes; (3) small genome size has evolved twice within Tetraodontiformes, once in smooth puffers and once in balistoids, thereby providing an opportunity to study the parallel evolution of small genome size in closely related lineages; and (4) a smooth puffer, *Fugu rubripes,* is being used as a model organism for gene identification and a plan to sequence the full genome of *F. rubripes* has been announced (http://fugu.hgmp.mrc.ac.uk). Pufferfish genomics may provide important information for understanding the evolution of genome size in these fishes.

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