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A Low Genomic Number of Recessive Lethals in Natural Populations of Bluefin Killifish and Zebrafish

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Despite the importance of selection against deleterious mutations in natural populations, reliable estimates of the genomic numbers of mutant alleles in wild populations are scarce. We found that, in wild-caught bluefin killifish *Lucania goodei* (Fundulidae) and wild-caught zebrafish *Danio rerio* (Cyprinidae), the average numbers of recessive lethal alleles per individual are 1.9 (95% confidence limits 1.3 to 2.6) and 1.4 (95% confidence limits 1.0 to 2.0), respectively. These results, together with data on several *Drosophila* species and on *Xenopus laevis*, show that phylogenetically distant animals with different genome sizes and numbers of genes carry similar numbers of lethal mutations.

One of the key genetic processes in any population is the dynamic equilibrium between the mutational origin of deleterious alleles and their eventual elimination by selection. Deleterious alleles maintained by mutation-selection balance create mutation load and contribute to genetic polymorphism and inbreeding depression (*1*, *2*). There are very few estimates of the genomic numbers of even those kinds of deleterious alleles that can individually produce drastic phenotypes and are readily detected by simple genetic assays for lethal and visible recessives. In several *Drosophila* species, the average number of visible alleles per individual ranges from 0.3 to 1.3 (*3*–*5*), and the number of lethals R is 1.0 to 3.0 (*6*–*11*). In *Xenopus laevis*, the only vertebrate for which there are published data, $R \sim 1.9$ (*12*).

Here, we report data on R in natural populations of two phylogenetically distant teleost fishes, *Lucania goodei* and *Danio rerio*. Both *L. goodei* and *D. rerio* were sampled from large wild populations in their native ranges, and a classical design (*3*) was used to detect their recessive alleles. We produced F_1 sibships by individual matings between different wild-caught parents. Within each sibship, individual brother-sister crosses yielded F_2 offspring, which were scored for mutant phenotypes, identified by abnormal morphology. Twenty-five percent of the lethals present in the wild-caught parents will be exposed as homozygotes in offspring from each F_1 cross. When this happens, 25% of the F_2 offspring show the effects of the recessive allele.

For the *L. goodei* experiment, we produced 20 F_1 sibships (*13*) and performed between one and four brother-sister crosses within each sibship. A total of 43 F_1 sib crosses was performed, and 30 to 90 offspring were examined from each cross, resulting in a total of 2569 F_2 offspring, 465 (18%) of which expressed mutant lethal phenotypes. No recessive lethals were found in 17 crosses, and 39 recessive lethals with clear-cut abnormal morphology were observed in the remaining 26 crosses (*14*). The fraction of abnormal F_2 embryos or fry was consistent with a 3:1 Mendelian ratio in all crosses except three, where it was too high. In two of these cases, the proportion of mutant phenotypes was explained better by hypothesizing that two unlinked loci act to produce the same effect. In contrast, only 8 (1.6%) out of 499 F_2 offspring, produced in nine control crosses between F_1 individuals from different sibships, were morphologically abnormal.

Thirty-one different lethals (18 before hatching and 13 after hatching) were found in 20 parental pairs (Table 1). Seventeen prehatching lethals could be subdivided into four clear phenotypic classes. We called them "early death," "worm," "pinhead" (which had several variants), and "bulbous head with beady eyes." They were clearly manifested (*14*) at 1 to 2, 4 to 7, 6 to 9, and 7 to 10 days after fertilization, respectively (Fig. 1). None of these mutants hatched. Twelve lethals that acted after hatching (12 to 14 days after fertilization) could be subdivided into four clear phenotypic classes called "humped," "curly," "uptail," and "no body pigment."

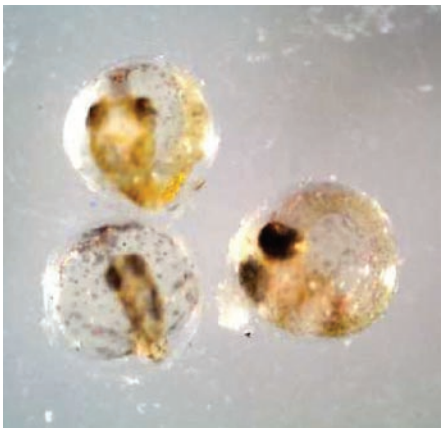

Fig. 1. Mutant and normal phenotypes for *Lucania goodei*. The phenotypes shown were obtained in cross 15b at 8 days after fertilization. The embryo on top is categorized as bulbous head with beady eyes. The embryo on the bottom left is a pinhead with bulbous ventricles. The embryo on the bottom right is wild type.

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Most humped, uptail, and no body pigment mutants failed to eat and died within 14 days of hatching. Curly mutants ate food but also died within 2 to 3 weeks after hatching. No lethals acting later than 14 days after hatching were found. Because mortality not associated with clear-cut abnormalities was low both in outbred control crosses and in sib crosses (*14*), our sample probably did not contain morphologically cryptic lethals. A recessive viable mutation was probably present in one sib cross, in which 3 out of 13 offspring that started eating could not properly control their swim bladders and floated beneath the surface tension of the water.

For the *D. rerio* experiment, we produced 13 independent F_1 sibships (*15*) from *D. rerio*, collected from the Ganges River drainage near Calcutta, India. Within each sibship, we obtained between 3 and 11 brother-sister matings. Of 86 sib crosses, yielding a total of 5696 F_2 offspring, 45 crosses revealed a total of 60 recessive lethal or visible mutations. No mutations were revealed, during 6 days of observation, in the remaining 41 crosses, yielding 1989 F_2 offspring (*16*). We found a total of 26 different recessive lethal mutations and 1 visible mutation carried by the 26 parental individuals (Table 2). Offspring with abnormal morphology were very rare (<1%) in 16 interfamily control crosses.

Mutant phenotypes (Fig. 2) (*16*) fell into four broad classes. Cranial mutations, found in 10 families, included those affecting head shape, eyes, and jaws [*ballooneyes*, *knucklehead*, *large lens*, *punchout*, *pseudopunchout* (Fig. 2), *knockout*, *blockhead*, *jawless eyebulgers*, *stifffjaw*, and *jaw deformity*]. Most cranial mutations were very distinctive morphologically. Three mutations (*punchout*, *knockout*, and *pseudopunchout*), from families 11, 10, and 8, respectively, appeared to be the same morphologically. However, three interfamily crosses between F_1 individuals, which must be heterozygous for a punchout-like mutant (since they previously produced a *punchout*-like phenotype in full sib crosses), produced only wild-type offspring. Thus, these mutations complement each other and must affect different loci.

The second class of mutations, found in nine families, involved loss of a swim bladder (*kinky*, *bottom heavy*, *bent*, *bladderless*, *sinker*, *lead weight*, *ballast*, *extra bubbles*, and *anchor*). Although various other mutants also lacked a swim bladder, these bladderless mutants have no other obvious abnormalities. A sample of 9 (out of 36 possible) interfamily crosses produced only wild-type phenotypes. Thus, bladderless phenotypes in different families are genetically distinct. An additional swim bladder mutant, in which the bladder was overinflated from days 6 to 9 before returning to normal size, occurred in one family. We classed *big bladder* as a visible mutant because several individuals survived longer than 21 days. In nature, however, this mutant would probably be lethal, given that such individuals floated at the surface.

The third class of mutations, axial abnormalities [*spirograph* (Fig. 2), *whirly*, and *candy cane*], occurred in three families. A complementation test of the phenotypically similar *spirograph* and *whirly* again demonstrated these mutations to be genetically distinct. Two mutations affected the gut region: one was the unique *darkguts*, and the second was *ventral edema*, found in two families but shown to be distinct with a complementation test.

Zebrafish mutations were generally first observed during the first 5 days of development, resulting in death considerably later, during days 6 to 15. Only one mutation, *dividing unevenly*, which was lethal within the first day of development, resulted in death before hatching. Fewer than half of the mutations detected were observed by day 3. Thus, it would have been impossible to obtain our estimate of *R* using haploid zebrafish, which can be scored for only the first 2 to 3 days after fertilization (*17*). In contrast to

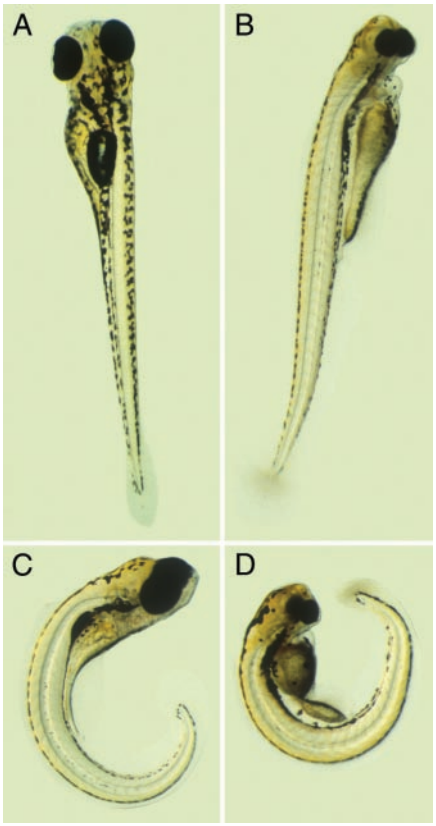

Fig. 2. Normal and mutant phenotypes of *Danio rerio*. Phenotypes shown are from a single sib cross (sibship 8, cross 5), which exposed two different recessive lethal phenotypes, *pseudopunchout* and *spirograph*. (**A**) Normal phenotype; (**B**) *pseudopunchout* individual with small eyes, knobby head, and no swim bladder; (**C**) *spirograph* individual with curved spine; and (**D**) an individual expressing both *pseudopunchout* and *spirograph* phenotypes simultaneously. Both mutant phenotypes appeared in expected Mendelian ratios relative to normal phenotypes in that clutch.

Table 1. Recessive lethals in 20 sibships of *Lucania goodei*. R_i is the estimated number of recessive lethals per parent (*21*).

Sibship	Number of unique mutations	Number of sib crosses	R_i	Mutant phenotypes (number of sib crosses in which phenotype was detected)
1	0	2	0	None
2	2	3	1.94	worm (1), pinhead (2)
3	1	2	0.87	humped (1)
4	1	2	0.87	curly (1)
5	2	1	3.74	worm (1), bent/humped (1)
6	3	2	2.80	pinhead (1), beady eyes (1), humped (1)
7	3	3	2.46	bulbous head with beady eyes (1), humped (3)*
8	1	2	0.87	humped (1)
9	2	3	1.16	pinhead (1), humped (1)
10	1	4	1.00	pinhead with corkscrew tail (1)
11	0	1	0	None
12	2	4	1.30	bulbous pinhead (2), pinhead with blood in tails and eyes (1)
13	0	1	0	None
14	3	1	5.75	early death (1), worm (1), uptail/humped (1)
15	2	2	4.78	pinhead with bulbous ventricle (2),*† bulbous head with beady eyes (2)
16	1	2	0.87	worm (1)
17	2	1	3.74	no body pigment (1), humped (1)
18	5	4	3.38	worm (1), pinhead with eyes posterior (2), humped (2),*† uptail (1)
19‡	0	1.5‡	0	None
20	0	1	0	None

*The proportion of animals with this phenotype was significantly higher than 0.25 in one cross within this sibship. †In one cross, the proportion of animals with this phenotype was explained significantly better by a model with two independent lethal alleles with this phenotype. ‡The same male was used in two sib crosses.

data for *L. goodei*, where nearly two-thirds of detected lethals acted before hatching (9 to 11 days after fertilization), a much larger fraction of zebrafish lethals acted after hatching.

The frequency of a mutant phenotype within a sib cross deviated significantly from the Mendelian expectation for 8 out of 60 mutations (*16*), including 6 mutations in the bladderless class found in four sibships. This may be due to incomplete penetrance, because frequencies of abnormal phenotypes in these crosses, although below 25%, were still much higher than any background rate of abnormality in control outbred crosses (*16*). Similar bladderless mutants were observed in screening for induced mutations (*18*). Several other mutants, such as *large lens*, *stifffjaw*, *spirograph*, and *whirly*, also resemble mutants found in artificial mutagenesis screens (*18–20*).

For each species, we estimated R, the mean number of lethals carried by a parent, by maximum likelihood (*21*). For *L. goodei*, $R = 1.87$, with 95% confidence limits 1.28 to 2.61. For *D. rerio*, $R = 1.43$ (95% confidence limits 0.95 to 2.04). With $R \sim 1$ to 2, mining of natural populations can feasibly complement artificial mutagenesis as a source of mutant alleles for studies in developmental biology. Most individuals carry one or two unique mutations, a number that makes mutant alleles easy to extract. Producing lethal-free lines from pairs of wild-caught fish is also feasible, because ~10% of such pairs should be lethal-free. Consistent with expectation, no lethals were detected in zebrafish sibship 6 (*16*).

Vertebrate species are strikingly similar in estimates of R. In this study, we estimate that $R = 1.87$ for *L. goodei* and that $R = 1.43$ for *D. rerio*. Previous work on *X. laevis* estimated that $R = 1.9$ on the basis of data from eight females, where both gynogenetic and inbred offspring were screened for mutants (*12*). It has also been suggested (*12*) that $R = 1.6$ in *Ambystoma mexicanum*, though this estimate is not recoverable from the original papers (*22*, *23*) cited in (*12*). This suggests that R may be rather uniform within vertebrates. Moreover, estimates of $R = 1$ to 2 from vertebrates are in the middle of the range of R in various *Drosophila* species and populations (*6–11*).

Data on R in other taxa are scarce. In one individual of loblolly pine, *Pinus taeda*, three to six lethals were detected (*24*). A larger R was reported in the Pacific oyster *Crassostrea gigas* (*25*), but the method used did not allow discrimination between true lethals and other causes of departures from Mendelian ratios that were common in these families. Data on mortality after inbreeding in mammals and other species where developing offspring receive nutrients from the mother can only provide an upper limit to R (*26*), because high mortality, not associated with any drastic mutations, is observed in even outbred offspring (*26*, *27*). R is distinct from the number of lethal equivalents [i.e., the number of lethals that would be required to explain the reduction in fitness after inbreeding, which takes into account the effects of mildly deleterious recessive alleles (*28*)].

The uniformity of reliable estimates of R is perplexing because there are reasons to expect much more variable estimates. For example, R might be scaled to genome size. The taxa for which we now have data on R vary by >100-fold in genome size [296×10^8 nucleotides in *A. mexicanum* (*29*), 61×10^8 in *X. laevis* (*30*), 18×10^8 in *D. rerio* (*31*), 11×10^8 in *L. goodei* (supporting online text), and 1.8×10^8 in *Drosophila melanogaster* (*32*)], and yet estimates of R are relatively constant. However, genome size does not measure the length of protein-coding sequences, which may be more relevant to R. Indeed, over 90% of phenotypically drastic mutations in humans occur within coding regions, whereas deleterious mutations in noncoding DNA are mostly mild (*33*).

Alternatively, R may be expected to scale to the number of genes in the genome, but this is also not the case. The number of genes in vertebrates is at least two to three times the number of genes in *Drosophila* [*D. melanogaster* has ~14,000 genes (*32*), *Homo sapiens* has at least ~35,000 genes (*34*), and genomes of mammals and of teleost fishes are highly syntenic (*31*, *35*)], and yet estimates of R do not vary by two- to threefold. This implies that the per locus frequency of recessive lethals in vertebrates may be one-half to one-third that in *Drosophila*. Given that the per locus mutation rates appear to be similar in vertebrates (*Mus* and *Homo*) and *Drosophila* (*36*), this suggests that heterozygous lethals persist for a shorter time in vertebrates.

A possible reason for the difference in per locus frequency of recessive lethals may be greater inbreeding in vertebrate populations. For example, inbreeding is probably responsible for the lower genomic number of recessive chlorophyll deficiencies in plants that undergo selfing (*37*, *38*). However, data on the complementation of lethals from different sibships of *D. rerio* and *L. goodei* suggest that, as in *D. melanogaster* (*6*, *8*, *10*, *11*), individual lethals are rare, which indicates a high effective population size. Furthermore, the population density of *L. goodei* is high, and our source population is itself very large (*39*); the same is probably true for wild zebrafish populations. Thus, there is no evidence of inbreeding in our source populations. Still, even weak inbreeding, which we cannot rule out, might purge recessive lethals to some extent (*37*).

How else do we explain the apparent constancy of the estimated values of R? One possibility is that selection against heterozygous lethals is stronger in vertebrates than in *Drosophila*. However, there are no data to support this hypothesis. Alternatively, the fraction of essential loci that can produce lethal effects may be lower in vertebrates, because of a greater overlap in gene function. However, the limited data available suggest that in both *Drosophila* (*32*) and vertebrates (*19*), the proportion of essential loci is ~20%. Ultimately, estimates of mutation rates, genomic numbers of recessive lethals, and numbers of essential loci from a variety of other natural populations will resolve this puzzle.

Table 2. Recessive lethals in 13 sibships of *Danio rerio*. R_i is the estimated number of recessive lethals per parent (*21*).

Sibship	Number of unique lethal mutations	Number of sib crosses	R_i	Mutant phenotypes (number of sib crosses in which phenotype was detected)
1	3	8	1.52	*ballooneyes* (4), *kinky* (2), *darkguts* (1)
3	1	6	0.50	*knucklehead* (1)
4	1	5	0.50	*bottom heavy* (1)
5	1	9	0.58	*large lens* (3)
6	0	6	0	None
7	1	3	0.58	*bent* (1)
8	3	5	3.57	*pseudopunchout* (4), *ventral edema* (3), *spirograph* (2)
9	1	9	0.50	*bladderless* (2)
10	2	5	1.74	*knockout* (1), *sinker* (3), *big bladder* (1)*
11	2	6	1.59	*punchout* (6), *lead weight* (1)
13	5	7	2.78	*blockhead* (1), *ballast* (2), *whirly* (4), *jaw deformity* (2), *bloated* (1)
14	5	11	2.50	*dividing unevenly* (1), *jawless eyebulgers* (1), *candycane* (2), *extra bubbles* (5), *stiffjaw* (1)
15	1	6	0.58	*anchor* (1)

*Visible mutation.

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21. Likelihood analysis was carried out on the vector of counts of lethals observed in each of the J F_1 crosses in the ith sibship, $\mathbf{r}_i = [n_{i1}, n_{i2}, \ldots, n_{ij}]$. R_{io} lethals were observed in the ith cross within each of the I sibships in each species. The log likelihood of a hypothesized mean lethal number R was calculated as

$$\ln L(R) = \sum_{i=1}^{I} \ln \sum_{R_i = R_{io}}^{\infty} P(R_i \mid R)\, P(\mathbf{r}_i \mid R_i)$$

where $P(.\mid.)$ is the conditional probability of the data. Each n_{ij} was assumed to be drawn from a binomial distribution out of an unknown number of lethals R leading to

$$P(\mathbf{r}_i \mid R_i) = \prod_{j=1}^{J} \frac{R_i!}{\mathbf{r}_{ij}!(R_i - r_{ij})!}\, 0.25^{r_{ij}} 0.75^{R_i - r_{ij}}$$

The distribution of lethal numbers in outbred parents was assumed to be Poisson, with parameter R, so

$$P(R_i \mid R) = \frac{e^{-2R}(2R)^{R_i}}{R_i!}$$

To find the value of R that maximized ln $L(R)$, we numerically solved $[\delta \ln L(R)]/\delta R = 0$ for R. The values in Tables 1 and 2 were calculated to maximize $P(\mathbf{r}_i \mid R_i)$.
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40. M. McNeilly helped collect eggs during the *L. goodei* experiment. M. McClure and K. Whitlock generously shared their expertise on maintaining, breeding, and rearing zebrafish. We are grateful to J. Birdsley, S. Ellner, N. Hairston, R. Harrison, and D. Winkler for helpful discussion and/or comments on the manuscript. We thank C. Kearns for advice on a variety of issues; K. Loeffler for help with photography; and R. Carlson, B. Diamond, M. Duffy, O. Duren, T. Sanger, W. Savage, K. Smith, and S. Williams for assistance with zebrafish care. Work on *L. goodei* was funded in part by an NSF dissertation improvement grant to R.C.F. and J.T. (DEB 00-73896) and a grant to J.T. (DEB 99-03925). Funding for the zebrafish work was provided by the NSF (DEB 9981445) to A.R.M.

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AGTR2 Mutations in X-Linked Mental Retardation

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Two angiotensin II (Ang II)–specific receptors, AGTR1 and AGTR2, are expressed in the mammalian brain. Ang II actions on blood pressure regulation, water electrolyte balance, and hormone secretion are primarily mediated by AGTR1. The function of AGTR2 remains unclear. Here, we show that expression of the *AGTR2* gene was absent in a female patient with mental retardation (MR) who had a balanced X;7 chromosomal translocation. Additionally, 8 of 590 unrelated male patients with MR were found to have sequence changes in the *AGTR2* gene, including one frameshift and three missense mutations. These findings indicate a role for AGTR2 in brain development and cognitive function.

Mental retardation (MR) affects 2 to 3% of the human population. Although several causative genes have been identified (*1*), the etiology of MR remains poorly understood. Alterations in molecular pathways involved in neuronal functions, especially cognition, are likely to play an important role.

Angiotensin II (Ang II) and related components of the renin-angiotensin system are largely known for their role in the regulation of blood pressure and water electrolyte balance, and antagonists of the Ang II receptor, as well as angiotensin converting–enzyme inhibitors, are effective therapeutics for hypertension (*2*). This action of Ang II peptide is primarily mediated by one receptor, AGTR1 (*3*). In contrast, the function of a second receptor, AGTR2, which has comparable affinity for Ang II, remains largely unknown. A possible role for AGTR2 in the central nervous system was suggested by the attenuated exploratory behavior and anxiety-like behavior of AGTR2-deficient mice (*4*–*6*). Results presented here support a role for AGTR2 in human cognitive function.

Linkage of several X-linked MR (XLMR) families to a large genetic interval, Xq23–q25, suggests the presence of one or more MR genes in the region (*1*). One, the *PAK3* gene, has been found to be mutated in MRX30 and MRX47 (*1*). To identify other candidate MR genes, we analyzed a de novo balanced translocation [46, X, t(X;7)(q24;q22)] in a female patient (DF) with moderately severe MR (with an intelligence quotient of 44). The MR phenotype in patient DF is likely due to skewed X inactivation that results in inactivity of both copies of an X-chromosomal gene, one on the inactive normal X chromosome and one on the active translocated X chromosome. We confirmed this idea, determining that X inactivation is totally skewed for one X chromosome (maternal) in patient DF, as compared to random X inactivation in her normal mother (*7*).

We mapped the X-chromosome breakpoint between markers DXS1220 (centromere) and DXS424 (telomere), developing a map of the breakpoint region (fig. S1) (*8*, *9*). Fluorescence in situ hybridization (FISH) with a series of genomic clones from the mapped region (fig. S1) enabled us to map the Xq24 breakpoint to a single P1–artificial chromosome (PAC) clone (dA5O9; approximately 115 kb) (Figs. 1A

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A Low Genomic Number of Recessive Lethals in Natural Populations of Bluefin Killifish and Zebrafish

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Editor's Summary