Annual Fish As a Genetic Model for Aging

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Advancement in the genetics of aging and identification of longevity genes has been largely due to the model organisms such as Caenorhabditis elegans and Drosophila melanogaster. However, knowledge gained from these invertebrates will not be able to identify vertebrate-specific longevity genes. The mouse has a relatively long life span of about 3 years, which limits its utility for screening of longevity genes. Fish have been used in aging studies. However, systematic comparison of survivorship curves for fish is lacking. In this study, we compared the survivorship curves of zebrafish and 2 different annual fish, namely, Cynelebias nigripinnis and Nothobranchius rachovii. These studies established that Nothobranchius rachovii has the shortest life span (8.5 months, at which time 10% of population remains). We also established that it is possible to breed Nothobranchius rachovii under laboratory conditions, and showed that their embryos can be stored for several months and hatched at any time by adding water. In addition, we have isolated 31 cDNA markers out of 71 attempted amplifications based on corresponding homologous genomic sequences in zebrafish and Fugu available from public databases, suggesting that approximately 40% of the genes from Nothobranchius rachovii could be easily isolated. Thus, the ability to be bred under laboratory conditions and the availability of cDNA markers for mapping, along with the major advantage of a relatively short life span, make Nothobranchius rachovii an attractive vertebrate genetic model for aging over other available vertebrate models.

MODEL systems such as Caenorhabditis elegans and Drosophila melanogaster have been used to study the genetics of aging and to identify genes involved in life span extension (longevity genes) (1–4). Nevertheless, these invertebrate models cannot identify longevity genes that are unique to vertebrates. Mice have been used in age-related studies such as identification of prolonged life span under dietary restriction and increased genomic instability during aging (5,6). Several theories, such as the role of reactive oxygen species, have been tested using mouse model systems (7,8). However, mice have a relatively long life span (3 years) and are not easily amenable to large-scale mutagenesis-recessive screens to identify longevity genes (9,10). Therefore, a vertebrate organism that has a shorter life span would be a better model for aging studies that is applicable towards understanding the genetic mechanisms of longevity in man.

Earlier studies in fish, such as oxidative stress as a causative agent of senescence, the protective role of antioxidant enzymes, decrease in ability to repair DNA, and retarded aging under dietary restriction conditions, showed that fish age in a way similar to that in other vertebrates (11–14). However, fish have not been used to identify longevity genes. Zebrafish have features such as transparency of the body, ability to perform saturation mutagenesis, generation of diploids from haploid eggs, and significant similarities of several physiologic processes to those found in humans for use as a vertebrate genetic model to study development and disease (15–18). However, the current study, as well as studies from other laboratories, have shown that the life span for zebrafish is about 3 years, thus limiting the use of zebrafish as a genetic model to study longevity genes in vertebrates (19,20).

Cynelebias nigripinnis and Nothobranchius rachovii are 4–5 centimeters in size and are commonly known as annual fish (also called killifish) because hobbyists have noted that these fish live for 1 year in aquaria. Under natural conditions, their life expectancy is thought to be 6 to 8 months. In Cynelebias adloffii and Nothobranchius guentheri, senescent changes such as atrophy of the thymus have been observed at around 4 months of age. An increased incidence of cancer is noted prior to the end of their less-than-1-year longevity (21). Despite the above use in aging research and anecdotal information on life span, no systematic study on their life spans has been undertaken, except for one report on survivorship curves using small numbers of Cynelebias bellottii showing their life span to be 16 months (22). Information on genes and markers for chromosomal mapping is also currently lacking in these annual fish.

In this article, we have compared the survivorship curves of zebrafish, Cynelebias nigripinnis, and Nothobranchius rachovii and demonstrated that Nothobranchius rachovii has a short survival time under stagnant water conditions and an even shorter survival time under recirculating water conditions. We have established that embryos could be hatched under laboratory conditions. Randomly chosen primers from regions of zebrafish expressed sequence tags (ESTs) homologous to Fugu gene sequences were used in polymerase chain reaction (PCR) amplifications, which revealed that approximately 40% of the gene markers corresponding to zebrafish could be identified in Nothobranchius rachovii. These studies mark the beginning of...
genetic studies on *Nothobranchius rachovii* and should be useful in identifying vertebrate-specific genes for longevity using saturation mutagenesis.

**METHODS**

**Aquaculture**

Zebrafish (4 months old) were purchased from Ekkwill Waterlife Resources (Gibsonston, FL) and *Cynolebias nigripinnis* (1 month old) and *Nothobranchius rachovii* (1 month old) species were purchased from Hawaiian Marine Enterprises (Kahuka, HI), who supplied hatching and spawning dates. For zebrafish, 200 fishes per 20-gallon tank were maintained with continuous ultraviolet (UV) light-treated water circulation. For killifishes, up to 4 fish were kept in 2-gallon tanks at 28°C under both circulating water and stagnant water conditions. Reverse osmosis-treated water was used and water quality was maintained by changing water once a week. Fish were fed frozen brine shrimp and UV-irradiated fish flakes twice daily. For breeding, *Nothobranchius rachovii* were fed live black worms purchased from Aquatic Foods (Fresno, CA), and embryos were collected in peat moss purchased from a local vendor, stored in a dry container for 4 months, and hatched by being placed in water. In vitro fertilization was performed according to the procedure described earlier (23).

**Survivorship Curves**

Fish counts were taken daily and survivorship curves were generated by plotting the percentage of survivors against time in months. Statistical distribution analysis of survivorship curves was obtained by using Minitab version 13.0 statistical software (Minitab, Inc., State College, PA). Median, mean, and confidence intervals were determined by using the nonparametric distribution analysis test with right censoring. Log-rank test was used for *p* value determination of significance of survivorship curves.

**Isolation of cDNAs by Amplifying the cDNAs Using Primers From the Zebrafish Genome**

Every EST from the 25 linkage groups of zebrafish, which are available in zebrafish linkage map databases (http://134.174.23.167/zonrhmapper/), was aligned with corresponding *Fugu* sequences (http://bahama.jgi-psf.org/fugu/bin/blast.fugu.cgi) in search of homologous regions. The sequences of these regions were used in designing primer sets that would result in an amplified product length between 200 and 250 bp. Total RNA was isolated from both *Cynolebias nigripinnis* and *Nothobranchius rachovii* and used in reverse transcriptase-PCRs to amplify cDNAs corresponding to zebrafish ESTs. Products were resolved on 2% agarose gels to estimate sizes of amplified DNA and cloned into pCR 2.1-TOPO vector and sequenced (24).

**RESULTS**

Since zebrafish are an excellent genetic model used for the study of development and disease, we initially wanted to investigate the life span of zebrafish and to test whether they can be used as a model for aging. Four-month-old zebrafish (*n* = 400) were placed in a 20-gallon tank, and the numbers of surviving fish were plotted over a 4-year period. The survivorship curve is shown in Figure 1. The longest surviving zebrafish was 45 months old with a median and mean life span of about 31 months. The 10% of population remaining (decile) life span was 41 months. The statistics of distribution analysis of the survivorship curve is given in Table 1. Another independent study was published (20) when this work was in progress (19) and found that mean life span for outbred zebrafish was 42 months, while for the golden sparse strain of zebrafish was 36 months. Taken together, the results suggested that zebrafish are not an ideal model for studies involving genetics of vertebrate aging due to their relatively long life span.

To investigate whether killifish would be suitable for aging studies, *Cynolebias nigripinnis* and *Nothobranchius rachovii* were chosen, since hobbyists have claimed that they have a relatively short life span and there are no careful studies on the life span of these fishes. Therefore, in our first experiment, 1-month-old *Nothobranchius rachovii* (*n* = 497) were introduced into a recirculation water system under similar conditions used in maintaining zebrafish (25). No more than 4 fish were maintained in each 2-gallon tank. The survivorship curve under these conditions gave a mean life span for *Nothobranchius rachovii* of 1.9 months with the longest-living fish living until 4.5 months of age. This is in contrast to that observed by the hobbyists who claimed the life span of this fish to be 1 year (Figure 2). The distribution of the survivorship curve for *Nothobranchius rachovii* under water recirculation conditions is shown in Table 1. This surprising result led to the hypothesis that the *Nothobranchius rachovii* may age faster under stress due to continuous water recirculation, which generates gas bubbles that may lead to gas bubble disease as observed in other fishes (26). It is also known that these fish live in stagnant ponds in nature. Therefore, in the second experiment, *Nothobranchius rachovii* (*n* = 69) and *Cynolebias nigripinnis* (*n* = 91) were placed in a stagnant water environment and water was changed manually only once a week. Under these conditions, the life span of these fishes was much greater (Figure 2) compared with our previous results. Comparison of the distribution analysis of survivorship curves of the *Cynolebias nigripinnis* and *Nothobranchius rachovii* revealed that
Nothobranchius rachovii had a shorter life span (decile life span of 8.5 months) than Cynolebias nigripinnis (decile life span of 9.7 months) (Figure 2, Table 1). We plotted survivorship curves for males and females of Cynolebias nigripinnis and Nothobranchius rachovii and found that there was no significant variation in life span between males and females (Figure 3). We also observed that the aged fish (8 months) compared with young fish (3 months) showed signs of reduced movement.

To test whether storing and hatching of embryos under laboratory conditions was possible, embryos (Figure 4) were collected in peat moss for 1 week, partially dried on paper towels, placed in a plastic bag, and then stored in a cool dry container. Females lay their eggs almost every day, and the yield of the embryos was approximately 20–30 per clutch per day. After 3 to 4 months, water was added to the stored embryos, and hatched embryos were observed 24 hours later (Figure 4). In vitro fertilized embryos also gave similar results. The transparency of the Nothobranchius rachovii embryo is not the same as zebrafish, but blood circulation and heartbeat are easily visualized (27). Also, the adult male and female Nothobranchius rachovii are easy to distinguish by color (Figure 4), in contrast to sexing of zebrafish, which is often relatively difficult. Once the bright colors are developed, the fish have reached adulthood and are ready to breed (approximately 1 month after hatching). Unlike zebrafish, where approximately 50%–60% of embryos

<table>
<thead>
<tr>
<th>Species of Fish</th>
<th>D. reria Male</th>
<th>N. rachovii Male</th>
<th>N. rachovii Female</th>
<th>C. nigripinnis Male</th>
<th>C. nigripinnis Female</th>
<th>C. nigripinnis Male</th>
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<tbody>
<tr>
<td>Statistical Parameters</td>
<td>Median (mo)</td>
<td>31.0</td>
<td>5.8</td>
<td>5.4</td>
<td>5.7</td>
<td>1.8</td>
</tr>
<tr>
<td>95% CI of median (mo)</td>
<td>31.45–31.55</td>
<td>6.15–6.45</td>
<td>5.70–6.06</td>
<td>6.07–6.31</td>
<td>2.18–2.27</td>
<td>7.11–7.46</td>
</tr>
<tr>
<td>Mean (mo)</td>
<td>32.2 (±0.3)</td>
<td>6.0 (±0.3)</td>
<td>5.6 (±0.4)</td>
<td>5.7 (±0.3)</td>
<td>1.9 (±0.1)</td>
<td>6.5 (±0.4)</td>
</tr>
<tr>
<td>95% CI of mean (mo)</td>
<td>31.5–32.8</td>
<td>5.8–6.6</td>
<td>4.7–6.4</td>
<td>5.2–6.2</td>
<td>1.8–1.9</td>
<td>5.8–7.2</td>
</tr>
<tr>
<td>10% of population remaining (mo)</td>
<td>41.0</td>
<td>8.3</td>
<td>8.4</td>
<td>8.5</td>
<td>1.9</td>
<td>9.1</td>
</tr>
<tr>
<td>Longest surviving fish (mo)</td>
<td>45</td>
<td>9.2</td>
<td>9.2</td>
<td>9.2</td>
<td>4.5</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Notes: *Kept in recirculation system. CI = confidence interval; D. = Danio; N. = Nothobranchius; C. = Cynolebias.

Figure 2. Survivorship curves of Nothobranchius rachovii in water recirculation system (solid circles), and Nothobranchius rachovii (solid diamonds) and Cynolebias nigripinnis (open circles) in stagnant water. The p value for Nothobranchius rachovii and Cynolebias nigripinnis curves (stagnant water) is .0005.

Figure 3. Survivorship curves of (A) Cynolebias nigripinnis (p value = .44) and (B) Nothobranchius rachovii (p value = .709) males (solid diamonds) and females (open circles).
survive to reach adulthood, nearly 90% of the *Nothobranchius* embryos survive and reach adulthood.

It is usually difficult to clone a gene from one species even though sequence of the homologous gene is available from another species. To test the number of homologous markers that can be isolated from *Nothobranchius rachovii* genome based upon gene information available in zebrafish and *Fugu* genome databases, we synthesized 71 primer pairs derived from randomly chosen ESTs obtained from zebrafish genome databases that were found to be conserved between zebrafish and *Fugu* sequences. The primers were used for RT-PCR amplifications using total RNA from *Cynolebias nigripinnis* and *Nothobranchius rachovii*. We found that 31 of these primer pairs gave amplified products according to predicted sizes in zebrafish for *Nothobranchius rachovii*, and 33 of these primer pairs gave products for *Cynolebias nigripinnis* (Table 2). Several of these primer sets amplified cDNA in both species while others were unique to each species. The primer sets that gave positive results and the corresponding zebrafish EST clone with their positions on the zebrafish chromosomes are listed in Table 2. Sequencing these products revealed that the amplified products yielded sequences corresponding to the zebrafish EST sequences. A representative sequence comparison of zebrafish ESTs and the corresponding *Nothobranchius rachovii* sequences is shown in Figure 5.

**DISCUSSION**

We have determined the life spans of zebrafish, *Cynolebias nigripinnis*, and *Nothobranchius rachovii* under carefully controlled conditions, which should reveal differences in life spans. We found *Nothobranchius rachovii* is the shortest lived among these fish. The embryos are easy to collect, in vitro fertilization is possible, and the embryos hatch and reach maturity in 1 month. Sexing of males and females is simple due to differences in colors. Due to the ease of isolation of genetic markers based on other fish genome information, genotyping for linkage analysis is feasible. Thus, these assets make *Nothobranchius rachovii* an excellent vertebrate genetic model for studying longevity genes.

Laboratory conditions such as temperature, water quality, as well as species and strain, appear to have an influence on the life span of the fish. For example, the mean life span of an outbred population of zebrafish was reported to be 42 months, whereas we used an Ekkwill strain (supposedly an inbred strain) that showed a mean life span of 32 months (20). Similarly, our study on *Cynolebias nigripinnis* showed a life span of 10 months, while studies by Liu and Walford on *Cynolebias bellottii* yielded a life span of 16 months (22). Thus, the observed differences may be due to strain or species variation, which is commonly seen in other organisms. Another major difference in life span was noted between water recirculation (1.9 months) and stagnant water...
It is remarkable that the zebrafish survivorship curve resembled a reasonably rectangular curve similar to what is observed in mice survivorship curves, suggesting that the early life mortality is low. Interestingly, Liu and Walford (22) also obtained similar rectangular shape survivorship curves for *Cynolebias*. Nevertheless, the shape of the survivorship curves for zebrafish seems to vary from a reasonably rectangular shape to close to a linear shape in earlier studies (20). In our studies on *Notobranchius rachovii* and *Cynolebias*, the curves were also close to linear in contrast to the zebrafish survivorship curves. The linearity suggests the possibility that mortality may be due to factors other than aging, such as the presence of infectious disease. Although we did not find any evidence for infectious disease, we cannot exclude such diseases because our fish were obtained from a commercial vendor. In this context, it is important to note that even under the best breeding and maintenance conditions, on average, only 50%–60% of zebrafish embryos survive, which may stress the fish and induce gas bubble disease, as has been reported in other fish (14). Thus, such variability also exists in mice due to cage variability. Duplicate and/or triplicate experiments should eliminate the effects of such minor variations. However, there is also a possibility of unexpected introduction of flora. If identical water conditions are required, the tanks could be connected and water could still be stagnant. Unfortunately, since the tanks are connected, such a scenario also has the risk of spreading potential infectious agents, which may escape UV treatment to multiple tanks.
reach adulthood. Thus, it is difficult to attribute the linearity of the survivorship curves to infectious disease and other non-age-dependent variables in fish, and such variations in curve shape may be natural for fish aging.

As is indicated in the Results section, we noted sluggishness in old fish. Even though we have not provided any quantitative data for measuring such activity, studies from other annual fish indicate that senescent changes (alterations in spinal curvature) suggest that there are age-related changes in annual fish (22). We are currently monitoring fish movement using a video surveillance system to correlate the activity of fish with age.

Often the isolation of a gene from one species has been difficult even when the sequence of its homologous gene is known in a different species. Our data revealed that it is possible to use primers derived from public databases of the zebrafish and *Fugu* genomes to isolate homologous markers in *Nothobranchius rachovii* in at least one third of the cases. The ease of procuring genetic markers in *Nothobranchius rachovii* will facilitate linkage mapping studies. Since such sequence homology exists among these fish species, further utilization of oligonucleotide-based microarrays could be used to rapidly identify several genetic markers and genes from *Nothobranchius rachovii*. This conservation may also be useful in analyzing age-specific gene expression in *Nothobranchius rachovii* using zebrafish microarrays. Furthermore, the fact that *Nothobranchius rachovii* females can lay as many as 20–30 eggs per clutch per day and both males and females reach sexual maturity at approximately 1 month indicates that breeding this fish for linkage analysis of long life-span mutant fish should be possible. Thus, these studies form the foundation for the use of *Nothobranchius rachovii* as a model organism for genetic studies in aging.

**Summary**

Our data established that *Nothobranchius rachovii* has a short life span and is easy to breed. Isolation of genetic markers for *Nothobranchius rachovii* by utilizing the zebrafish and *Fugu* genomes is relatively easy. Thus, in the future, these fish could be subjected to ethyl-nitroso urea saturation mutagenesis and progeny could be selected for long life span. Sperm could be collected from the males and
preserved until males with the longest life span have been identified, and then could be used to recover progeny. Similarly, eggs collected from females could be fertilized in vitro and kept for a few months until the identification of the longest-lived female. These long life-span fish could then be propagated to identify vertebrate-specific longevity genes by linkage analysis.

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