

TEMPO OF HYBRID INVIABILITY IN CENTRARCHID FISHES (TELEOSTEI: CENTRARCHIDAE)

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Abstract.—Hybrid viability decreases with divergence time, a pattern consistent with a so-called speciation clock. However, the actual rate at which this clock ticks is poorly known. Most speciation-clock studies have used genetic divergence as a proxy for time, adopting a molecular clock and often far-distant calibration points to convert genetic distances into age. Because molecular clock assumptions are violated for most genetic datasets and distant calibrations are of questionable utility, the actual rate at which reproductive isolation evolves may be substantially different than current estimates suggest. We provide a robust measure of the tempo at which hybrid viability declines with divergence time in a clade of freshwater fishes (Centrarchidae). This incompatibility clock is distinct from a speciation clock because speciation events in centrarchids appear to be driven largely by prezygotic isolation. Our analyses used divergence times estimated with penalized likelihood applied to a phylogeny derived from seven gene regions and calibrated with six centrarchid fossils. We found that hybrid embryo viability declined at mean rate of 3.13% per million years, slower than in most other taxa investigated to date. Despite measurement error in both molecular estimated ages and hatching success of hybrid crosses, divergence time explained between 73% and 90% of the variation in hybrid viability among nodes. This high correlation is consistent with the gradual accumulation of many genetic incompatibilities of small effect. Hybrid viability declined with the square of time, consistent with an increasing rate of accumulation of incompatibilities between divergent genomes (the snowball effect). However, the quadratic slope is due to a lag phase resulting from heterosis among young species pairs, a phenomenon rarely considered in predictions of hybrid fitness. Finally, we found that reciprocal crosses often show asymmetrical hybrid viabilities. We discuss several alternative explanations for this result including possible deleterious cytonuclear interactions. Speciation-clock studies have been a small cottage industry recently, but there are still novel insights to be gained from analyses of more taxonomic groups. However, between-group comparisons require more careful molecular-clock calibration than has been the norm.

Key words.— F_1 asymmetry, hybridization, hybrid viability, molecular clock, reproductive isolation, speciation clock.

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Due to its gradual nature, the process of speciation usually cannot be followed from inception to completion in a researcher's lifetime. To circumvent this limitation, studies of speciation often take a comparative, cross-sectional approach. Rather than follow the branching of a single lineage through time, one can survey a number of species pairs that have progressed different distances along the path toward speciation. Assuming that the different pairs follow similar evolutionary trajectories caused by similar mechanisms, one can obtain general observations about the tempo and mode of speciation.

An example of this cross-sectional approach is the venerable observation that the capacity to hybridize decreases as one moves from closely to distantly related pairs of taxa. Darwin (1859; p. 257) noted that "the fertility of first crosses between species, and of the hybrids produced from them, is largely governed by their systematic affinity. This is clearly shown by hybrids never having been raised between species ranked by systematists in distinct families; and on the other hand, by very closely allied species generally uniting with facility." Darwin's anecdotal observation has since been supported by quantitative comparative studies of reproductive isolation. The basic approach to documenting this pattern is exemplified by Coyne and Orr's (1989, 1997) seminal studies of reproductive isolation in *Drosophila*. Using allozyme di-

vergence (Nei's *D*) as a measure of evolutionary distance between taxa, Coyne and Orr plotted the degree of pre- and postzygotic isolation against the genetic distance separating pairs of species. Both components of isolation accumulate steadily with genetic distance and so presumably with time.

The positive relationship between isolation and genetic distance has been repeatedly confirmed by comparative studies in other taxa (Edmands 2002; Coyne and Orr 2004), including angiosperms (Moyle et al. 2004), Lepidoptera (Presgraves 2002), sea stars (Foltz 1997), fish (Mendelson 2003; Russell 2003), anurans (Sasa et al. 1998), and birds (Price and Bouvier 2002; Tubaro and Lijtmaer 2002; Lijtmaer et al. 2003). This pattern has come to be known as the "speciation clock" (Coyne and Orr 1998), though this term may be something of a misnomer. The majority of speciation-clock studies focus on intrinsic postzygotic isolation. If these incompatibilities mostly arise after other forms of reproductive isolation, these clocks may actually have very little to do with speciation. Coyne and Orr (2004, p. 57) argue that "speciation involves the study of isolating barriers only up to the point at which gene flow between the taxa is close to zero, but such barriers continue to accumulate thereafter." We therefore use the term "incompatibility clock" throughout this paper. However, incompatibilities are not completely unrelated to speciation, even if they arise after behavioral or ecological barriers are complete. This is because genetic incompatibilities buttress diverging species against introgres-

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sion following speciation should the environment change in a way that eliminates any behavioral or ecological barriers.

Despite the consensus that a rough incompatibility clock exists, the rate and pattern of reproductive isolation appears to vary among *Drosophila*, anurans, and lepidopterans (Edmunds 2002; Mendelson et al. 2004), and among mammals, birds, and frogs (Wilson et al. 1974; Fitzpatrick 2004). However, these among-clade comparisons are complicated by the fact that different studies use different currencies to measure evolutionary distance. To date, genetic divergence is the most widely used metric of evolutionary distance in studies of speciation clocks. The molecular data required to calculate genetic divergence are now readily available for many groups of organisms, facilitating comparative studies of speciation (Mendelson et al. 2004). There is also a good biological justification for measuring rates in terms of genetic distance: genetic divergence between taxa is thought to have a causal, mechanistic relationship to reproductive isolation that other measures, such as taxonomic rank or number of generations, lack (Fitzpatrick 2002). However, measures of genetic divergence are not easily compared across studies. Substitution rates vary among clades and along lineages, and studies differ in the type of data used to measure genetic divergence. A given percent sequence divergence (or Nei's *D* value) may not be comparable between *Drosophila* and frogs. This is particularly true when genetic distances are calculated using different genes (or types of genetic data) that are likely to evolve at very different rates (Gillespie 1991).

A common solution is to convert genetic divergence into a more comparable and intuitive currency, namely time. This conversion requires a molecular clock, which assumes that substitution rates are roughly constant so that genetic distances are proportional to time (Sanderson 1998). Unfortunately this assumption is rarely warranted, as substitution rates commonly vary among lineages (Langley and Fitch 1974; Britten 1986; Gillespie 1991; Sanderson 1998, 2002). Although rate heterogeneity is the rule rather than the exception, none of the studies of the incompatibility clock tested for it. Recently, a number of tools have been developed to compensate for rate heterogeneity, greatly facilitating the estimation of divergence times using molecular data (Sanderson 1997, 2002; Huelsenbeck et al. 2001).

Rates of molecular evolution must also be correctly calibrated per unit time, using either geological events or fossils to assign ages to particular points in the phylogeny. Calibration errors can occur if fossils are incorrectly dated or assigned to the wrong part of the tree (Near et al. 2005). Errors are even more likely when calibration points or substitution rates are taken from far-distant taxa rather than within the focal group of organisms (which may have different substitution rates). Lacking fossils within *Drosophila*, Coyne and Orr (1989) were obliged to transform allozyme-derived Nei's *D* into time by assuming a rate calibrated for mammals (Nei 1987). Of the many recent comparative studies of the rate of reproductive isolation, only Price and Bouvier (2002) and Fitzpatrick (2004) used a fossil calibration within the focal clade. The tempo of speciation clocks is therefore poorly known, due to a frequent failure to account for rate heterogeneity or provide appropriate calibrations in converting

relative age inferred from genetic data to absolute evolutionary age.

In this paper, we take advantage of robust divergence time estimates and published data on hybrid inviability to document the tempo of reproductive isolation in Centrarchidae. Centrarchids are a clade of 32 species of freshwater fishes including sunfish, crappies, rock basses, and black basses that are endemic to North America. Near et al. (2004, 2005) provide a well-supported fossil-calibrated phylogenetic hypothesis for all 32 described species of centrarchids, using sequence data from seven gene regions (three mitochondrial and four nuclear). Integrating analyses to correct for molecular evolutionary rate heterogeneity (Sanderson 1997, 2002, 2003) with the extensive centrarchid fossil record, Near et al. (2005) derived unusually well-supported estimates of centrarchid divergence times. Regressing hybrid viability on these divergence time estimates allows us to calibrate the incompatibility clock in centrarchids, measured in real time rather than genetic distance. In addition to providing a more robust estimate of the rate at which hybrid inviability evolves, we discuss several insights into the mechanisms of species divergence, including Haldane's rule, the snowball effect, and possible causes of asymmetrical F_1 viability from reciprocal crosses.

METHODS

Divergence Time Estimates

Our incompatibility clock is based on absolute age measures of divergence time, in millions of years. The details of these estimates are described fully in Near et al. (2005), but we give a brief overview of these methods here. The centrarchid phylogeny is based on the sequences of four nuclear genes (*S7* intron 1, calmodulin intron 4, *Tmo4C4*, and rhodopsin) and three mitochondrial sequences (ND2, 16S rRNA, and a set of three tRNAs: Met, Trp, Ala), for between one and three individuals from all 32 described species of centrarchids. These genes represent between two and five unlinked loci. The phylogeny was estimated with a partitioned mixed-model Bayesian analysis (Ronquist and Huelsenbeck 2003) using Metropolis-coupled Markov chain Monte Carlo implemented in Mr. Bayes 3.0 (Larget and Simon 1999; Huelsenbeck et al. 2001). Optimal substitution models were identified with ModelTest 3.0 (Posada and Crandall 1998). The molecular phylogeny was calibrated using six centrarchid fossils that yield mutually consistent age estimates (Near et al. 2005), and penalized likelihood was used to allow for variation in rate parameters across the tree. The computer program r8s (ver. 1.6) was used for all divergence time estimates (Sanderson 2003).

Measures of Interspecific Divergence

We surveyed the literature on centrarchid hybridization to collect published information on the degree of reproductive isolation between species pairs (see Appendix available online only at <http://dx.doi.org/10.1554/04-563.1.s1>). While some data are available on most components of pre- and postmating isolation (fertilization rates; embryo, larval, and juvenile viability; hybrid fertility; hybrid mating success; F_2

viability; F₂ fertility), only hybrid embryo viability is published for enough crosses to justify a comparative study. The patterns described in the remainder of this paper therefore focus on this one component of reproductive isolation.

In all cases, published embryo viability data were generated through artificial laboratory crosses. Experimenters manually stripped eggs from gravid females and mixed the eggs with sperm stripped from males to produce hybrid embryos. The percent of embryos surviving to larval stage was recorded for both hybrid crosses, and for homospecific crosses. The homospecific cross is required to control for low egg viability that may result from the stripping process. A few studies failed to provide control cross data, and were excluded from our analysis. While many sources do not report the actual number of eggs used in crosses, those that do tend to range from the hundreds to several thousand for a given cross (see online Appendix).

While few studies report fertilization rates (indicated by cleavage after 1 h), those that do make it clear that fertilization rates remain high (>90%) for nearly all centrarchid crosses (West and Hester 1966; Merriner 1971a). While we feel confident that low hatching success indicates hybrid inviability rather than a failure of fertilization, it is possible that some of our results conflate these factors. We also note that while natural centrarchid hybrids are not uncommon, it is unlikely that researchers mistakenly used hybrids for crosses, as hybrids are readily recognizable (Hubbs 1944; Neff and Smith 1979).

Hybrid viability (or compatibility) was calculated as the percent of hybrid embryos that hatched into larvae, relative to the hatch rate of a homospecific control cross:

$$\text{compatibility} = 100 \times \frac{\% \text{ heterospecific hatching}}{\% \text{ homospecific hatching}} \quad (1)$$

(Ralin 1970; Sasa et al. 1998; Mendelson 2003). Compatibility close to 100% indicates little or no postzygotic isolation, while a compatibility approaching zero indicates strong isolation. Compatibility can exceed 100% if hybrids have higher viability than single-species embryos. Compatibility is inversely related to the amount of postzygotic isolation, so our data could also be presented with an index of inviability (100 – compatibility). Where a given species pair cross had been replicated multiple times, we averaged the results of multiple experiments to yield a single measure of postzygotic compatibility.

Averaging all compatibility measures for a given species pair obscures one important source of variation, asymmetry in crossing success. Asymmetry occurs when the viability of hybrids between two species is different depending on which species is the maternal parent. To account for this variation, we calculated the relative asymmetry of reciprocal crosses as

$$\text{asymmetry} = 1 - \frac{\text{compatibility}(A \times B)}{\text{compatibility}(B \times A)}, \quad (2)$$

where B × A is the more successful cross direction. An asymmetry of zero indicates equal crossing success for reciprocals. In addition to this relative measure, we calculated the absolute difference between reciprocal cross compatibilities.

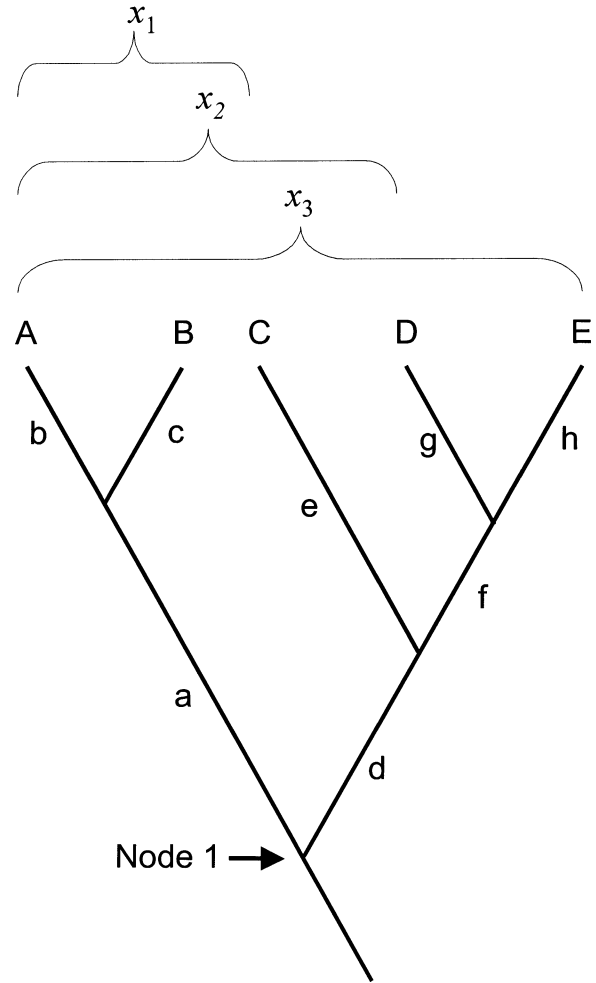


FIG. 1. A schematic diagram illustrating the statistical nonindependence of three crosses, x_1 , x_2 , and x_3 due to shared evolutionary history (see text). Species are identified by capital letters, branch lengths are represented by lowercase letters.

Nonindependence of Pairwise Relationships

A measure of divergence between a given pair of species (i.e., reproductive isolation or Nei's D) may not be independent of pairwise relationships among other pairs of species. To illustrate this point, consider the hypothetical phylogeny in Figure 1. Shared evolutionary history will likely produce some correlation between hybrid viabilities of crosses A × C and B × D. This is because isolating mechanisms that arose before the nodes separating A and B or C and D (i.e., along branches a or d in Fig. 1) will affect hybrid viability in both crosses. Recognizing this problem, Coyne and Orr (1989) suggested treating the nodes as the level of replication. Calculating a mean degree of isolation for each node in the phylogeny, one would then regress mean isolation at a node against the node age. In the context of Figure 1, this means treating the mean viability of A × B hybrids as independent of crosses A × C, A × D, and A × E (which are averaged to get a viability for node 1), even though all crosses involve species A. This approach hinges on an untested assumption that the genes involved in incompatibilities between species A and B are different from the genes separating A and (C,

D, or E). If this assumption is violated, we will have overestimated the degrees of freedom in our analysis. Nonetheless, the node-contrast approach remains the best available method for studying the incompatibility clock in small clades where there are not enough fully independent species pairs whose connecting branches do not overlap.

Coyne and Orr (1989, 1997) calculated the mean pairwise divergence at a node by averaging the values for all species pairs that span a particular node. Unfortunately, this averaging process again ignores the effect of phylogenetic structure within nodes (Fitzpatrick 2002). Consider crosses x_1 , x_2 , and x_3 in Figure 1. Because taxa D and E share more evolutionary history with each other than they do with taxon C, we would expect crosses x_2 and x_3 to be more similar to each other than they are to cross x_1 . Simply averaging these three crosses will give excessive weight to divergence that occurred along branch f . The solution to this problem is to carry out a weighted averaging procedure (Fitzpatrick 2002) equivalent to Felsenstein's independent contrasts (Felsenstein 1985). The average of the three crosses would be:

$$\bar{x} = \frac{\frac{x_1 + x_2}{2} + x_3}{2} \quad (3)$$

instead of

$$\bar{x} = \frac{x_1 + x_2 + x_3}{3}. \quad (4)$$

Even this weighting scheme is problematic, as it fails to account for branch lengths. Equation (3) gives insufficient weight to the fraction of time in which taxa D and E were independent, while equation (4) discounts their shared history. We therefore used a weighted average that accounts for shared branch lengths of taxa used in the crosses for the node, measured as the duration of each branch in millions of years using our rate-smoothed and calibrated phylogeny. Given an ultrametric tree with known branch lengths (such as the chronogram used for this analysis, Fig. 2), we calculated the weighted mean isolation for a node as:

$$\bar{x} = \frac{\sum_k \left(x_k \sum_i \frac{L_i}{n_i} \right)}{\sum_j L_j}, \quad (5)$$

where there is data for k pairs of species subtending the node of interest, x_k is the measure of divergence between the k th pair of species, and L_i is the length of the i th branch of the phylogeny, where the i branches are the subset of the branches in the phylogeny connecting the two species involved in cross x_k . The set of j branches used to standardize equation (5) represent the set of all branches connecting taxa for which divergence data are available. For instance, in Figure 1, species B is not used for any comparisons, so we consider seven rather than eight branches, $L_j \in [a, b, d, e, f, g, h]$. The variable n_i is the number of distinct crosses that route through a particular branch of the phylogeny. Considering the three crosses in Figure 1, $n_a = n_b = n_d = 3$; $n_f = 2$, and $n_e = n_g = n_h = 1$. This equation provides an average pairwise distance for a given node, down-weighting pairs that share a large propor-

tion of their evolutionary history with other species pairs, and giving greater weight to crosses that are independent of other crosses for a larger proportion of their history. It is equivalent to branch-length corrected independent contrasts (Felsenstein 1985), modified for pairwise relationships between species. For comparison, we also ran all analyses with node-averages calculated using equations (3) and (4). Qualitative results and statistical significance were similar for all three approaches, indicating our results are robust to our choice of averaging procedure.

Regression of Isolation on Divergence Time

We used equation (5) to calculate phylogenetically weighted averages for hatching compatibility (eq. 1) and reciprocal cross symmetry (eq. 2) for all nodes in the phylogeny for which such data was available. We then regressed average divergence at each node against node age (Table 1, Near et al. 2005) to estimate the linear relationship between each variable and age. For compatibility data, we used both linear and quadratic regression models, both with and without forcing the regression through the 100% y -intercept (by definition, our measure of compatibility must be 100% for crosses between individuals that have not diverged at all). Partial F -tests determined whether quadratic terms significantly improved the fit of the model. Kolmogorov-Smirnov tests were used to check whether variables were normally distributed, and significance levels for all tests recalculated using non-parametric Spearman's rank correlation. As qualitative results were similar and normality was not rejected, we focus on the parametric statistical results.

Net Diversification Rate

We qualitatively assessed the constancy of the net diversification rate by generating a log-lineage through time plot. A linear increase in the logarithm of the cumulative number of species indicates a constant rate. The relatively small number of species precludes a robust quantitative test of rate constancy. Having determined the total branch length within the centrarchids (in units of millions of years), we then used the Kendall-Moran estimator to determine the net diversification rate, the difference between speciation and extinction rates (Baldwin and Sanderson 1998; Nee 2001). The inverse of this rate represents a lineage's mean waiting time between speciation events that lead to extant descendants. The inverse is an underestimate of the actual speciation rate, because it does not include the origination of now-extinct species.

RESULTS

The Rate of Evolution of Hybrid Inviability

We found data on hatching compatibility for 130 crosses from 37 pairs of species representing 12 nodes of the phylogeny (Fig. 2, Table 1, online Appendix). These crosses involved 17 of the 32 described species of centrarchids and are dominated by crosses involving four species: either *Micropterus salmoides*, *Micropterus floridanus*, *Lepomis gulosus*, or *Lepomis macrochirus* were one parent in half of the pairwise comparisons. Eight of the 12 nodes have data for a single species pair (Table 1), and in two cases only a single

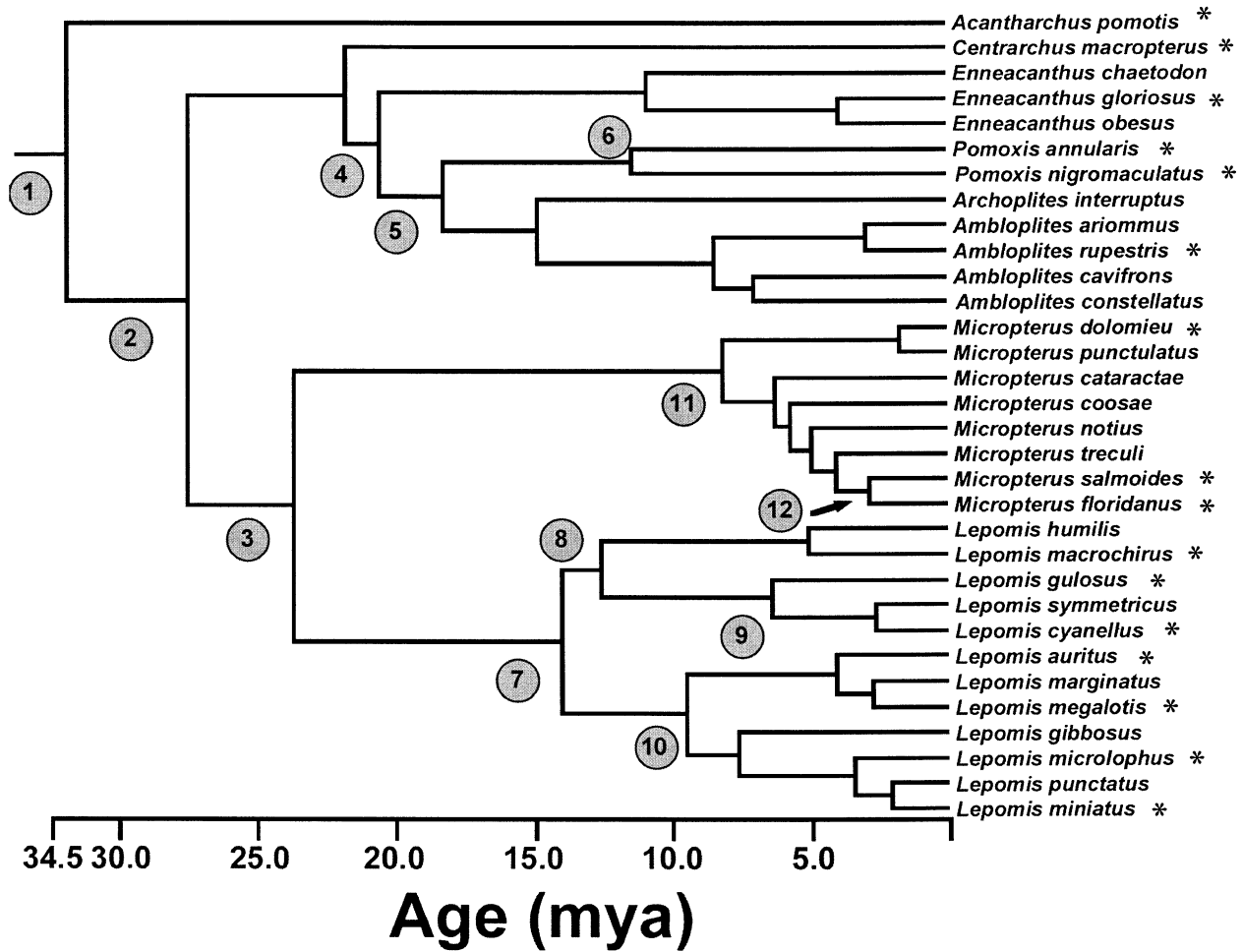


FIG. 2. The phylogeny of centrarchids, with branch lengths representing time in millions of years (adapted from Near et al. 2005). Nodes with data on hybrid viability are labeled, and species used for hybrid crosses are marked with asterisks. Table 1 provides information on each labeled node.

cross direction is available (no reciprocal). The uneven sampling of hybrid crosses in our dataset arises from several factors. First, much of the hybridization data is from aquaculturists, who are interested in a limited set of popular cen-

trarchids species. Second, many of the missing species are uncommon or have restricted ranges and so are more difficult to acquire. The low replication and lack of reciprocals at some nodes should increase the scatter in our regressions, so

TABLE 1. Information on each contrast (node) used in our analysis: number of species pairs with hybridization data at each node, node age, mean hatching compatibility, mean degree of reciprocal cross asymmetry (absolute difference in compatibility, and 1 – the ratio of less to more successful cross). Nodes are identified with numbers corresponding to labels in Figure 2.

Node	Number of species pairs	Node age (million years)	Mean hatching compatibility	Mean difference in compatibility of reciprocal crosses	Mean relative asymmetry of reciprocal crosses
1	1	33.59	26		
2	13	28.94	6.64	10.07	0.46
3	8	24.81	57.45	50.62	0.77
4	1	21.60	14.2		
5	1	19.18	71.07	45.55	0.47
6	1	12	87.45	14.85	0.21
7	5	14.64	103.66	20.01	0.25
8	2	13.11	76.43	14.93	0.35
9	1	6.6	101.5	33	0.08
10	1	9.81	85.5		
11	1	8.4	92	20	0.2
12	1	2.84	97.8	17.08	0.16

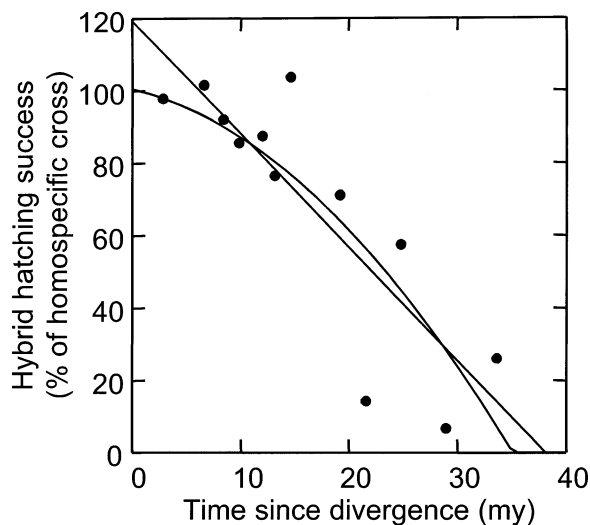


FIG. 3. Mean hatching compatibility of interspecific crosses as a function of node age. Compatibility is measured as the ratio of the percent hatching success of the heterospecific cross to the success of the homospecific cross. Lines indicate the quadratic regression forced through the y-intercept of 100% and the unforced linear regression.

we expect our results to be conservative relative to what we would see with a more complete dataset.

Linear regression shows that hybrid viability declined with the age of the node separating the parental species ($P < 0.001$; Fig. 3). Compatibility declined at a rate of 3.13% per million years from a starting value of 119.4% (Table 2). This suggests that on average reduced hatching success (compatibility < 100) did not begin to accumulate until taxa had diverged for 6.2 million years. While heterosis was observed in some relatively young crosses, the y-intercept should by definition be 100%, not 119%. It therefore appears likely that the age-isolation curve is nonlinear.

Quadratic regression failed to detect any significant curvature to the age-viability function or improve the overall fit of the model (Table 2). In contrast, when we forced the regression through a y-intercept of 100% compatibility for undiverged populations, quadratic regression did significantly improve the model. In the forced quadratic regression, the linear term was no longer significant and instead hybrid viability declined at an accelerating rate of 0.061% per (million years)² (Table 2). In contrast to the lag of 6.2 million years indicated by simple linear regression, the forced quadratic

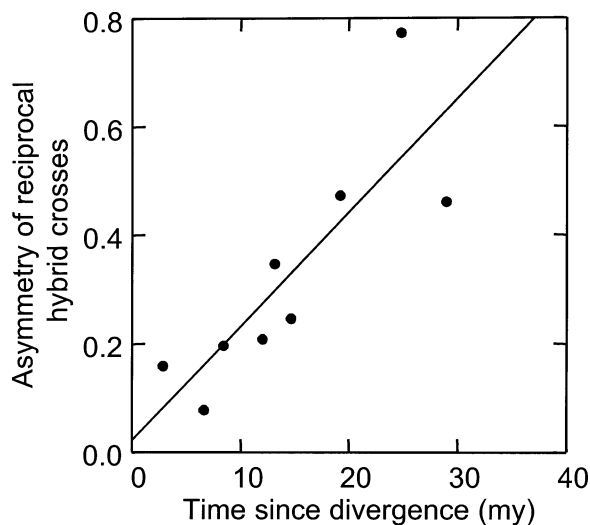


FIG. 4. Mean hatching asymmetry as a function of node age. Asymmetry is measured as $1 -$ the ratio of compatibilities of reciprocal crosses, with the less compatible cross as the numerator.

slope implied that hybrid viability began to decline immediately after initial divergence (the maximum of the quadratic curve was at 0.0 million years of divergence). Because the confidence intervals for the linear term included positive values, we could not reject the possibility of an initially positive slope indicative of a brief period of increasing heterosis before viability began to decline. Although the best-fit model had a uniformly negative slope, reproductive isolation accumulated quite slowly early on: it took 4.83 million years for compatibility to decline to 95%. The 6.2 million year lag time identified by the linear model corresponds to 93.1% compatibility.

The Rate of Evolution of Reciprocal Cross Asymmetry

Of the 37 pairs of species for which we had artificial cross data, 20 pairs had reciprocal crosses that allowed us to calculate the asymmetry of reciprocal crossing success. Two of these pairs had reciprocal crosses with zero compatibility in both directions, leaving us with 18 estimates of cross asymmetry through nine contrasts in the phylogeny. The relative asymmetry of reciprocal crosses increased with divergence time (Fig. 4; $t_9 = 4.03$, $P = 0.005$, $r^2 = 0.700$). The best-fit regression line (Symm = 0.02 + 0.021t; 0.09 and 0.005 intercept and slope SE, respectively) suggests that cross

TABLE 2. Results of four different regressions of mean hybrid viability (compatibility) at each node against node age. Models were either linear or quadratic and were either forced through a y-axis intercept of 100%, or were not forced. For each regression, we present the estimated intercept, linear slope with respect to time (age), and quadratic slope with respect to time (age²). Standard errors for the intercepts are provided in parentheses. Significance and *t*-values for the linear and quadratic terms are provided for each regression along with *r*²-values. Partial *F**-values test whether the quadratic term significantly improved the fit of the model.

Model	Forced	Intercept	Age	Age ²	Linear term		Quadratic term		<i>r</i> ²	<i>F</i> *	<i>P</i>
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>			
Linear	no	119.4 (11.2)	-3.13 (0.60)		-5.2	<0.001	—	—	0.730		
Quadratic	no	113.3 (21.38)	-2.24 (2.71)	-0.025 (0.07)	-0.83	0.429	-0.34	0.742	0.734	0.03	0.866
Linear	yes	100	-2.34 (0.22)		-10.43	<0.001	—	—	0.784		
Quadratic	yes	100	-0.74 (0.65)	-0.061 (0.024)	-1.13	0.266	-2.56	0.016	0.824	5.56	0.041

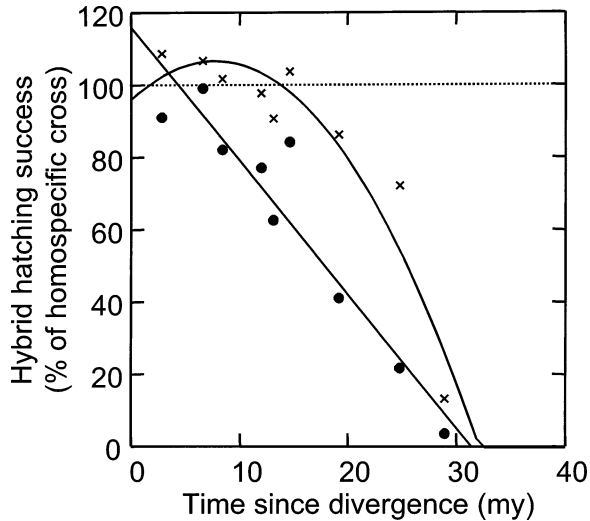


FIG. 5. Mean hatching compatibility of reciprocal interspecific crosses as a function of node age. The lower regression (circles) is the compatibility of the more successful reciprocal cross direction, and is best explained by a quadratic curve (time: $P = 0.206$; time²: $P = 0.022$; $r^2 = 0.90$). The upper regression line (crosses) is the compatibility of the less successful cross, best explained by a linear regression ($P < 0.001$; $r^2 = 0.90$; quadratic regression did not significantly improve the fit of the model: partial $F_{1,7} = 1.57$, $P = 0.25$).

asymmetry increases at a rate of about 2% per million years, distinctly slower than the linear rate of decline in hybrid viability (3.1%). Quadratic regression did not improve the fit of the model, whether or not we forced the regression through a y-intercept of 1.0. The absolute difference in hatching success did not show a significant relationship with node age ($t_9 = 0.671$, $P = 0.524$, $r^2 = 0.06$).

Another way of representing the evolution of asymmetry in reciprocal F_1 crosses is to simultaneously plot the viability of the more and less successful cross direction at each node (Fig. 5). By selecting the more and less successful crosses post hoc, we create one artifactual result: the regression line for the more successful crosses will by definition fall above that of the less successful cross. There are three useful observations that emerge from this exercise. First, asymmetries occur at nearly all nodes. Asymmetries were found in 17 of the 20 species pairs for which reciprocal crosses have been done. Two of the three symmetrical species pairs showed zero compatibility in both directions. Second, the vertical offset between the pairs of datapoints is actually fairly constant, consistent with our observation that the absolute magnitude of the asymmetry does not vary with node age. Instead, relative asymmetry increased because a given difference in compatibility represents a larger proportion as viability declines. Third, we see that when heterosis (viability $> 100\%$) occurs, it usually affects only one cross direction.

Net Diversification Rates

The plot of the cumulative number of lineages-through time is linear and so does not qualitatively suggest any rate variation through time (Fig. 6). The very slight upturn in diversification rate during more recent time is to be expected

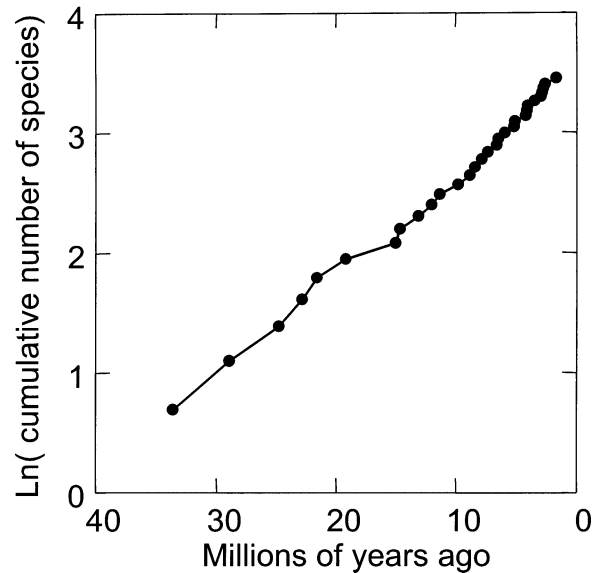


FIG. 6. Log-lineage through time to document variation in the net diversification rate through time.

with a constant diversification rate because some fraction of recently speciated lineages that will go extinct have not yet done so. Using a Kendall-Moran estimate of the net diversification rate based on a pure birth process (Baldwin and Sanderson 1998; Nee 2001), we found that the mean net diversification rate in centrarchids is 0.090 species per lineage per million years (95% CI: 0.066, 0.128), or an expected waiting time of 11.15 million years for a lineage to speciate into surviving descendents (95% CI: 7.76, 15.24). There was no statistically significant rate variation among the three major subclades of centrarchids, though power was limited. The expected waiting time for a lineage to split is more than twice as long as the average internode length (4.66 million years ± 0.65 SE), which is not surprising because internode length excludes long branches that have yet to speciate. The average age of extant sister species was 4.3 million years ± 1.0 ($N = 10$, range: 1.67–12 million years).

DISCUSSION

Centrarchids obey an approximate incompatibility clock, as hybrid viability declined with the time separating pairs of species (Fig. 3). This result is not unexpected, as similar trends have been described for a wide range of taxa (Coyne and Orr 1998, 2004; Edmands 2002). However, few studies have provided reliable estimates of the rate at which the speciation clock ticks. By taking advantage of robust divergence time estimates based on multiple fossil calibration points and relaxed-clock methods that account for heterogeneity in substitution rates (Near et al. 2003, 2005), we provide the most precise estimate to date of the tempo of an incompatibility clock. Our results provide several insights into mechanisms of postzygotic isolation.

Slow Loss of Hybrid Viability in Centrarchids

Using the available data, it appears that hybrid inviability accumulates roughly linearly in centrarchids at a rate of a

3.13% reduction in hatching success per million years. This slow loss of hybrid viability begins after an initial lag phase of approximately 6 million years of little or no inviability (e.g., *Micropterus floridanus* × *M. salmoides*, *Lepomis cyanellus* × *L. gulosus*). Recently diverged taxa may even show a degree of heterosis: *L. cyanellus* × *L. microlophus* hybrids showed a 13% higher hatching success than intraspecific crosses; *L. auritus* × *L. macrochirus* hybrids had 19% higher hatching success than their controls.

The long lag phase and slow decline in viability suggest that centrarchids evolve postzygotic isolation more slowly than many other taxa investigated to date. No other studies have measured the incompatibility clock in a comparable manner (percent loss of viability per million years). However, we can use several benchmarks as a basis for comparisons between groups. Setting aside questions about their clock calibrations, previous studies claim that the minimum age for total hybrid inviability is 1.5 million years in anurans (Sasa et al. 1998), 2 million years in *Drosophila* (Coyne and Orr 1997), 4 million years in Lepidoptera (Presgraves 2002), and 5.5 million years in birds (Price and Bouvier 2002; Lijtmaer et al. 2003). In contrast, the minimum age for total inviability in centrarchids is 24.81 million years (node 3, Fig. 2), though this is only for one direction of a reciprocal cross. The other direction of this cross (*L. microlophus* × *M. salmoides*) yielded 43% viability. The only crosses with total inviability in both directions are *M. salmoides* × (*Ambloplites rupestris*, *Pomoxis annularis*, or *Pomoxis nigromaculatus*) at 28.94 million years, while 10 other crosses of that age have some viability in one or both reciprocal directions (see online Appendix). Centrarchids also retain nonzero viability and heterosis for much longer than most other taxa.

We propose two possible explanations for why centrarchids evolve genetic incompatibilities more slowly than other taxa. First, the comparisons discussed above ignore the effect of generation time. With generation times of two to four years, it is not surprising that isolation would take longer for centrarchids than for *Drosophila*. However, this explanation is not completely satisfying, as centrarchids are also slower than the equally long-lived birds, mammals, and anurans. A more likely explanation is that Haldane's rule, an important mechanism of postzygotic isolation, may be weak or absent in centrarchids. Theory suggests that hybrid fertility and inviability should evolve more quickly in taxa with larger X chromosomes and slower in taxa with smaller ones (Orr and Turelli 2001). This is because the size of the sex-specific chromosomal region determines the number of hemizygous recessive alleles that can interact with dominant autosomal loci to produce hybrid dysfunction (Turelli and Begun 1997).

Karyotypic analysis of centrarchids failed to find karyotypically distinct sex chromosomes (Roberts 1964; but see Becak et al. 1966). A more recent study found evidence that centrarchid males are the heterozygous sex (Gomelsky et al. 2002), but did not assess whether this heterozygosity is limited to one or a few loci or extends to a large fraction of one of the 48 chromosomes (still a small fraction of the total genome). In some fishes the difference between male- and female-determining chromosomes is restricted to a few hundred kilobases or fewer of male-specific sequence (Kondo et al. 2003). The heterogametic sex is therefore hemizygous for

very few loci, reducing the potential for deleterious epistatic interactions between a recessive X allele and an autosomal locus (Turelli and Begun 1997; Orr and Turelli 2001). As a result, Haldane's rule will not apply in fish such as centrarchids with little or no hemizygous genome. Because the incompatibilities producing Haldane's rule are expected to evolve relatively quickly (Orr and Turelli 2001), and contribute strongly to postzygotic isolation in many groups (Coyne and Orr 2004), the absence of Haldane's rule in centrarchids may explain their slower evolution of genetic incompatibilities. It is telling that, of the many examples of Haldane's rule reviewed in Laurie (1997), none are drawn from fishes. Another genus of fishes, *Etheostoma*, also evolves inviability very slowly (Mendelson 2003) and lacks a distinct sex chromosome (Danzmann 1979).

The slow evolution of postzygotic isolation in centrarchids is accompanied by a relatively slow diversification rate. The mean diversification interval (the inverse of the net diversification rate) is 11.15 million years, slower than the diversification intervals for 38 of the 45 animal groups tabulated in Coyne and Orr (2004, pp. 419–420). Centrarchid diversification is slower than the measured rates in any other vertebrate clades except *Ictalurus* (North American catfish, which also lack a distinctive sex chromosome; LeGrande and Dunham 1984). While this measure underestimates the actual speciation rate (it excludes speciation events ending in extinction), it reinforces the impression of slow diversification in centrarchids, even relative to other vertebrates with long generation times.

Strong Correlation between Age and Hybrid Viability

There is appreciable measurement error for both the viability and age estimates. As emphasized above, we feel that our analysis has used the best currently available techniques to minimize error in measuring divergence time (Near et al. 2005). Nevertheless, there may be error in the phylogenetic topology and each node age comes with confidence intervals often several million years wide. The measures of hybrid viability are also subject to error, as illustrated by variation in hybrid viability within any given cross. For instance, *L. gulosus* × *M. salmoides* yields a mean compatibility of 102.5% (range 93–116%), and the reciprocal yields 75% (62–96%). Both cross directions are more compatible than the mean for that node (57%). This measurement error for a given cross likely reflects both chance variation and different crossing techniques among our sources. Biologically real variation among species within a node can arise from the stochastic nature of substitutions causing inviability, arising from either drift or selection on particular taxa. Finally, the *L. gulosus* × *M. salmoides* example illustrates the error in our dataset arising from species pairs with data for only a single cross direction. As this error is likely to be randomly distributed, we do not believe it biases our results, but it almost certainly leads to lower correlations than might otherwise be obtained.

Given these potentially substantial sources of error, it is impressive that divergence time explains such a large percentage of the variation in hybrid viability among nodes (73% for the linear regression; $r_s = -0.85$). Our explanatory power is even stronger when we focus on just the more or less

successful cross direction ($r^2 = 0.90$ in both cases). This suggests that some of the error in the overall regression is indeed caused by mixing crosses with and without reciprocals. Even so, our analysis yields a tighter time-viability relationship than any other incompatibility clock study, with the exception of Fitzpatrick's (2002) regression of postzygotic isolation against DNA hybridization distance in *Drosophila* ($r^2 = 0.786$). Other studies have found correlations ranging from 0.58 (Presgraves 2002) to 0.65 (Tubaro and Lijtmaer 2002). These lower correlations may reflect the studies' coarse categorical measures of reproductive isolation and their untested molecular clock assumptions.

The high correlation between divergence time and hybrid viability has two important implications for our understanding of species divergence. First, a high correlation lends further support to the idea that postzygotic isolation evolves through the gradual stochastic accumulation of many Dobzhansky-Muller incompatibilities with small effects (Orr and Turelli 2001; Coyne and Orr 2004). If inviability was generally due to the stochastic accumulation of a few large-effect incompatibilities, the regression should have a higher error and a paucity of datapoints with intermediate levels of viability. Conversely, when inviability depends on the cumulative effect of many small incompatibilities the age-viability correlation should be stronger.

Second, residual variation in Figure 3 can reflect real deviations in the incompatibility clock due to factors that accelerate (or slow) divergence. For instance, pairs of *Drosophila* species with higher-than-expected isolation also have high allozyme divergence, suggesting that divergent selection at the molecular level promotes inviability (Fitzpatrick 2002). In contrast, the tight time-viability relationship in Centrarchids leaves little residual variation to explain. Factors like selection may still be involved in driving divergence for particular genes (e.g., Presgraves et al. 2003; Barbash et al. 2004), but appear to have little effect on the overall rate of isolation. We have found no relationship between the level of range overlap or allozyme divergence and hybrid compatibility (D. I. Bolnick, unpubl. data).

Snowballing Inviability or Heterosis?

As noted above, there is a lag phase in the centrarchid incompatibility clock. Hybrids of recently diverged species (< 6 million years) are at least as viable as the within-species control crosses. So, while our actual data shows no significant curvature, the linear regression predicts a biologically untenable y-intercept of 119% viability for crosses between fish with zero divergence. We therefore stepped beyond the data to force the regression line through the logically necessary y-intercept of 100%. Quadratic regression then provides a better fit to the data, implying that hybrid viability declines with the square of divergence time. Similar curvature is seen in our regression of more successful crosses (Fig. 5), without forcing the intercept. This is the first incompatibility-clock study to find that inviability arises with the square of time, though Mendelson et al. (2004) showed that several other groups have a lag phase.

The quadratic loss of viability in centrarchids may be compatible with the snowball effect. Theory predicts that the

number of genetic incompatibilities will rise with the square of the amount of time separating two species (Orr 1995; Orr and Turelli 2001). While loci diverge at a steady rate with time, each new substitution has an increasing number of already-diverged loci in the other species with which it can produce incompatibilities. Empirical support for the snowball effect has been limited, as none of the earlier incompatibility-clock studies found significant quadratic curvature in their regressions (e.g., Lijtmaer et al. 2003). The lack of curvature in these comparative studies may not be informative, for two reasons. First, the snowball effect assumes there is no gene flow between taxa throughout divergence. If this assumption is violated, genetic incompatibilities should accumulate linearly instead (Kondrashov 2003). Second, the comparative studies use measures of hybrid inviability or reproductive isolation, whereas the theory is built on the number of genetic incompatibilities. Reproductive isolation will only snowball if inviability is a linear function of the number of incompatibilities. It is unknown whether incompatibilities have the requisite additive effects. Consequently, linear loss of viability cannot be used to reject the snowball effect.

A recent re-analysis of several speciation-clock datasets using a new likelihood approach has found that some taxa have a lag phase (as in centrarchids), consistent with snowballing reproductive isolation (Mendelson et al. 2004). Can lag phases or quadratic curves, as we find in centrarchids, be used to validate the effect? This depends on whether other processes can also give rise to a lag phase. We can think of two. First, if ancestral polymorphisms are sorted during speciation, the molecular branching points used in the x-axis of Figure 3 can predate the start of speciation when incompatibilities begin to arise. In this case, the lag reflects a period of prespeciation polymorphism at the marker used to date divergence. Given the low within-species genetic distances in centrarchids and the concordance between our multiple loci, this is not likely to explain the lag of 6 million years in centrarchids. Second, outcrossing benefits might lead to heterosis in hybrids between recently diverged taxa (Lopez et al. 2000). If this heterosis is strong enough to override early incompatibilities, a lag phase will result even if the incompatibility clock is linear. Consistent heterosis among young centrarchid taxa suggests that the quadratic incompatibility clock may result from initial outcrossing benefits rather than the snowball effect. One tantalizing observation is that more successful reciprocal cross directions show both heterosis and quadratic curvature, while less successful directions show neither (Fig. 5).

Asymmetries in F₁ Hybrid Viability

Centrarchid hybrid viability differs between reciprocal crosses of the same pair of species (F₁ asymmetry). Of 18 species pairs for which reciprocal crosses have been done (and viability is nonzero), 17 had significantly different viabilities depending on which species was the female (or male) parent. The relative strength of this asymmetry increased linearly with time (Fig. 4), because the absolute difference in viabilities was fairly constant and represented an increasing proportion of the overall viability as the latter measure declined. F₁ asymmetries have been documented in a wide range

of organisms, including *Drosophila* (Sturtevant 1920; Wu and Davis 1983) and numerous plant genera (Tiffin et al. 2001). Unfortunately, they have received no theoretical attention to date that might explain the regularities in the evolution of asymmetry that we see in centrarchids.

Dobzhansky-Muller incompatibilities are often asymmetrical (Welch 2004), as shown by reciprocal introgressions of single chromosome fragments (Wu and Beckenbach 1983; Orr and Coyne 1989; Elena and Lenski 2001). Under the standard model of incompatibilities, a population with genotype *aabb* diverges into *AAbb* and *aaBB*. Since alleles *A* and *B* have never coexisted, they may be incompatible and introgressing allele *A* into an *aaBB* background can result in low fitness. The reciprocal introgression of allele *a* into *AAbb* will not result in low fitness, because the combinations *Aa* and *ab* both existed during the evolutionary transition from *aabb* to *AAbb*. Theory therefore predicts that individual genes will produce asymmetrical introgressions. However, these asymmetries appear in backcrosses, not F_1 hybrids that have genotype *AaBb* regardless of which species is the maternal parent and so should have equal viability.

Dobzhansky-Muller incompatibilities can be asymmetrical when they involve an interaction between haploid and diploid loci. Let *m* and *n* represent mitochondrial (or hemizygous sex chromosome) and nuclear alleles respectively. When a population *mnn* splits into daughter species with genotypes *Mnn* and *mNN*, reciprocal crosses yield *MNN* (potentially incompatible) and *mnn* (ancestral) that are likely to be asymmetrical. Mitochondrial-nuclear incompatibilities have been demonstrated by experimentally inserting foreign mitochondria into a cell (Kenyon and Moraes Carlos 1997) and can be distinguished from haploid sex chromosome/autosomal interactions by the fact that the latter will only cause asymmetries in the hemizygous sex (Coyne and Orr 2004).

Asymmetrical F_1 viabilities may also result from deleterious interactions between the maternally provided oocyte cytoplasm and the hybrid's nuclear genes. Centrarchid hybrids show aberrant timing of allozyme gene expression during early development, even when the parental species have identical onset of gene expression (Phillip et al. 1983). These results suggest that centrarchid species have diverged in their gene regulation mechanisms even while expression location and timing remained similar. In many cases, hybrids expressed maternal alleles at the normal time, but paternally derived alleles were delayed, premature, or failed to be expressed at all (Phillip et al. 1983). Less viable hybrids in a reciprocal cross are generally the ones with greater paternal allele misexpression. Whitt et al. (1977) suggested that the greater effect on paternal alleles is evidence for cytoplasmic-nuclear interactions, hypothesizing that maternally encoded regulatory signals are misinterpreted by the paternal allele. If one species' gene expression is more sensitive to changes in transcription factors, asymmetries will result.

We do not currently have enough information to distinguish between sex chromosome, mitochondrial, or cytoplasmic effects. However, the lack of distinctive sex chromosomes (Roberts 1964; but see Becak et al. 1966) suggests that the hemizygous nuclear region is likely to be small (possibly even a single locus) and so may not contribute strongly to inviability (Turelli and Begun 1997). One puzzling pattern

to emerge from our data lends some credence to a role for cytonuclear interactions: using maximum body size as an index (Page and Burr 1991), the larger species tends to be the more successful maternal parent (Table 3). Of the 18 species pairs with reciprocal cross data and nonzero viability, one pair had equal body size and nearly symmetrical crossing success. Focusing on the remaining 17 species pairs (admittedly not phylogenetically independent; Table 3), the larger parent was more successful in 13 crosses and less successful in four crosses ($\chi^2_1 = 4.765$, $P = 0.029$). We speculate that there is greater disruption of paternal allele expression when the paternal allele is from a smaller species, placed in an egg with cytoplasmic factors encoded by a larger maternal species. However, the cytoplasmic effect cannot be attributed to differences in egg size, as egg size is not correlated with body size (D. I. Bolnick, unpubl. data) and egg size differences are not associated with inviability (Merriner 1971b). We are working on expanding our dataset to include more reciprocal crosses to test this pattern more rigorously.

Overall Reproductive Isolation in Centrarchids

The patterns of reproductive isolation documented in this paper focused exclusively on hybrid viability from the zygote through the larval stage. While this can tell us a lot about the origins of intrinsic genetic incompatibilities (i.e., their rate, number/size of incompatibilities, role of cytonuclear interactions), it may have very little to do with speciation. This is because hybrid inviability only contributes to reproductive isolation if habitat, temporal, behavioral, mechanical, and gametic isolation are incomplete (Coyne and Orr 2004), and if viable hybrids are fertile, ecologically fit, and capable of finding mates.

The average age of sister taxa and mean internode branch length (4.3 and 4.66 million years, respectively) are short relative to the time required to accumulate significant postzygotic incompatibilities. This makes it clear that hybrid inviability plays little role in speciation, so we conclude that speciation in centrarchids is largely a matter of either prezygotic isolation or hybrid infertility. Mate choice and prezygotic isolation have been studied intensively in *Lepomis* (Clark and Keenleyside 1967; Keenleyside 1967; Gerald 1971; Steele and Keenleyside 1971; Clark et al. 1984). Both males and females preferentially court and spawn with conspecifics based on visual cues such as earflaps (Childers 1967; Goddard and Mathis 1997), pharyngeal sound production (Gerald 1971; Ballantyne and Colgan 1978), and behavioral cues (Steele and Keenleyside 1971; Keenleyside 1967; Clark et al. 1984). Despite these ethological barriers, natural hybridization is common even among taxa that have been separated for up to 14.64 million years (Fig. 7). Individuals will actively court and spawn with heterospecifics (Clark et al. 1984) and respond to sounds produced by other species (Ballantyne and Colgan 1978). Premating barriers may be circumvented by sneaker males that steal copulations and hence are not subject to the same degree of female discrimination as nesting males (Jennings and Philipp 2002).

In centrarchids, postzygotic isolation can take the form of reduced hatching rates (Childers 1967), developmental abnormalities (Whitt et al. 1972), larval mortality (Childers

TABLE 3. Cross asymmetry and maximum body size for species pairs with reciprocal cross data. Node number corresponds to node labels in Figure 2. Mean compatibility is listed for each cross direction ($A \times B$ or $B \times A$), the range is indicated in parentheses for those crosses with more than one published value. Species that are the dam in the more successful reciprocal cross are listed as the better dam. For cases with unequal body size and nonzero hybrid viability in both directions, we indicate whether the better dam is also the larger parent. Where egg sample sizes were available, we used a chi-square analysis to test whether the asymmetry was significant at $P < 0.05$ (asterisk).

Species		Node	Compatibility				Body size		Better dam	Larger	Better = Larger?
			$A \times B$		$B \times A$		A	B			
A	B		Mean	Range	Mean	Range					
<i>Ambloplites rupestris</i>	<i>Lepomis gulosus</i>	2	0	—	5.5	(5–6)	43	31	<i>L. gul</i> *	<i>A. rup</i>	no
<i>A. rupestris</i>	<i>Lepomis macrochirus</i>	2	26	—	5	—	43	41	<i>A. rup</i> *	<i>A. rup</i>	yes
<i>A. rupestris</i>	<i>Micropterus salmoides</i>	2	0	(0)	0	(0)	43	97	both zero	<i>M. sal</i>	n/a
<i>A. rupestris</i>	<i>P. nigromaculatus</i>	5	41	—	86.5	(86–87)	43	49	<i>P. nig</i> *	<i>P. nig</i>	yes
<i>Lepomis cyanellus</i>	<i>L. gulosus</i>	9	99	(97–101)	107	—	31	31	<i>L. gul</i>	equal	n/a
<i>L. cyanellus</i>	<i>L. macrochirus</i>	8	105	(85–140)	90	(79–100)	31	41	<i>L. cya</i> *	<i>L. mac</i>	no
<i>L. cyanellus</i>	<i>Lepomis microlophus</i>	7	118	(111–125)	107	(96–118)	31	24	<i>L. cya</i> *	<i>L. cya</i>	yes
<i>L. cyanellus</i>	<i>M. salmoides</i>	3	11	—	80	—	31	97	<i>M. sal</i>	<i>M. sal</i>	yes
<i>L. gulosus</i>	<i>L. macrochirus</i>	8	77	(58–100)	35	(10–70)	31	41	<i>L. gul</i> *	<i>L. mac</i>	no
<i>L. gulosus</i>	<i>L. microlophus</i>	7	107	—	82	—	31	24	<i>L. gul</i> *	<i>L. gul</i>	yes
<i>L. gulosus</i>	<i>M. salmoides</i>	3	75	(62–96)	103	(93–116)	31	97	<i>M. sal</i>	<i>M. sal</i>	yes
<i>L. gulosus</i>	<i>Pomoxis nigromaculatus</i>	2	1.7	(0–5)	2.3	(0–4)	31	49	<i>P. nig</i>	<i>P. nig</i>	yes
<i>L. macrochirus</i>	<i>L. microlophus</i>	7	77	(63–99)	102	(91–110)	41	24	<i>L. mic</i> *	<i>L. mac</i>	yes
<i>L. macrochirus</i>	<i>M. salmoides</i>	3	7	(2–10)	70	(55–83)	41	97	<i>M. sal</i>	<i>M. sal</i>	yes
<i>L. macrochirus</i>	<i>P. nigromaculatus</i>	2	13	(11–18)	44	(39–48)	41	49	<i>P. nig</i>	<i>P. nig</i>	yes
<i>L. microlophus</i>	<i>M. salmoides</i>	3	0	—	43	—	24	97	<i>M. sal</i>	<i>M. sal</i>	yes
<i>Micropterus dolomieu</i>	<i>M. salmoides</i>	11	82	—	102	—	69	97	<i>M. sal</i>	<i>M. sal</i>	yes
<i>Micropterus floridanus</i>	<i>M. salmoides</i>	12	109	(96–121)	91	(69–112)	97	97	<i>M. flo</i> *	<i>M. sal</i>	yes
<i>M. salmoides</i>	<i>Pomoxis annularis</i>	2	0	—	0	—	90	53	both zero	<i>M. sal</i>	n/a
<i>M. salmoides</i>	<i>P. nigromaculatus</i>	2	0	(0)	0	(0)	90	49	both zero	<i>M. sal</i>	n/a
<i>P. annularis</i>	<i>P. nigromaculatus</i>	6	98	—	77	—	53	49	<i>P. ann</i>	<i>P. ann</i>	yes

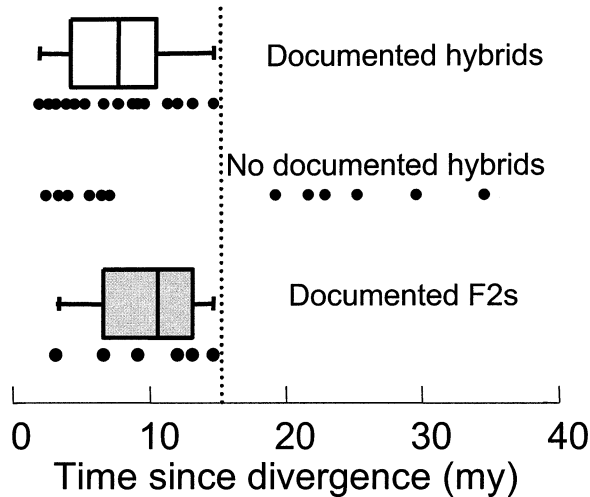


FIG. 7. Raw data (circles) and box plots showing the mean and confidence intervals of node age for nodes with documented natural hybrids (shaded box) and viable F₂s (open box, also implies fertile F₁s). Raw data for nodes without documented natural hybrids are also provided. The vertical dotted line indicates the minimum age difference between distinct genera.

1967), failure to develop gonads (West 1970), altered spawning behavior (Clark and Keenleyside 1967), meiotic failure leading to triploid progeny (Dawley et al. 1985), a biased hybrid sex ratio (Childers 1967), and inviable or infertile backcross or F₂ progeny (Dawley 1987). Gametic isolation does not appear to play a major role in isolation among centrarchids, as fertilization success is greater than 90% (relative to homospecific crosses) for nearly all nodes (West and Hester 1966; Merriner 1971a) and is not correlated with genetic divergence. Hybrid viability, analyzed in this paper, is thus an underestimate of the total postzygotic isolation. For instance, *L. macrochirus* × *L. cyanellus* show a 99% hatch rate relative to homospecific crosses (range: 79–140), and so do not appear to have begun to accumulate isolation. Yet from 68 to 97 percent of the progeny are male, with varying degrees of fertility due to a high frequency of unreduced (4n) sperm (Wills and Sheehan 2000). While hatching viability is greater than zero even at 33.59 million years, we have not been able to find any documentation of F₁ fertility for taxa more than 14.64 million years apart (Fig. 7). Note that the male-biased sex ratios in *Lepomis* hybrids do not provide evidence for Haldane's rule. Since *Lepomis* hybrids with close to 100% males show close to 100% viability, we can reject the idea that mortality of (hypothetically heterogametic) female zygotes produced the sex ratio.

Summary

Coyne and Orr spawned a small cottage industry of incompatibility-clock studies (Coyne and Orr 2004). While we need no further proof that incompatibility rises with divergence time, additional studies will continue to add insights on mechanisms driving this pattern, and provide the fodder for between-taxon comparisons of the rate of evolutionary divergence. Use of a common currency for divergence, properly calibrated, is necessary for future comparative studies of incompatibility clocks across taxa. Our primary goal for

this study was to raise the methodological bar by pointing out the importance of more sophisticated molecular-clock methods and careful calibration of molecular phylogenies. The application of relaxed-clock methods to a well-resolved multilocus molecular phylogeny that has been calibrated with a rich fossil record has allowed us to develop unusually precise estimates of the rate at which centrarchids accumulate genetic incompatibilities.

In addition to describing this improved methodology, our analysis uncovered patterns that shed light on broader issues. We speculate that the slow evolution of hybrid inviability in centrarchids reflects a lack of distinctive large hemizygous sex chromosomes, preventing the more rapid accumulation of inviability via Haldane's rule (Turelli and Begun 1997). The asymmetrical viability of reciprocal F₁ hybrids argues for a major role of interactions between haploid (sex or mitochondrial loci) and diploid genes, or cytonuclear interactions in postzygotic isolation. Previous studies of allozyme gene expression ontogeny in hybrid centrarchids lend support to the latter effect (Whitt et al. 1977). Our results also suggest that theory needs to assess the interaction between heterosis and genetic incompatibilities before we can judge whether a lag phase (Mendelson et al. 2004) in the evolution of hybrid inviability can be used to support the snowball effect (Orr and Turelli 2001). Finally, we argue that the evolution of hybrid inviability plays little role in driving speciation in centrarchids. Nonetheless, inviability may still play an important role in buttressing species against subsequent introgression. This is because other reproductive isolating mechanisms can break down under habitat disturbance or environmental change, whereas intrinsic genetic incompatibilities guarantee that diverged lineages remain distinct.

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Appendix

Collected published data on hybrid viability in centrarchids. The maternal parent in each cross is indicated with an asterisk (*). Nodes are identified with numbers corresponding to node labels in Figure 2. Hybrid viability (compatibility) is expressed as the percent of hybrid progeny hatching, divided by the hatch rate of a homospecific control cross. Crosses listed only once were not necessarily unreplicated, as most authors have lumped multiple crossings into a single value for a species pair, while other studies listed replicate crosses separately. We also present the number of eggs used to generate hybrids for a given cross. Where egg numbers were not available (n/a), the authors had presented data either as a percentage or as compatibility.

Cross		Node	Compatibility (%)	Number of eggs	Reference
<i>Acantharchus pomotis</i>	<i>Micropterus floridanus</i> *	1	26	n/a	Parker et al. 1985b
<i>Ambloplites rupestris</i>	<i>Enneacanthus obesus</i> *	4	14	123	Tyus 1973
<i>Ambloplites rupestris</i>	<i>Lepomis gulosus</i> *	2	5	315-11,109	Hester 1970
<i>Ambloplites rupestris</i>	<i>Lepomis gulosus</i> *	2	6	3943	Tyus 1973
<i>Ambloplites rupestris</i> *	<i>Lepomis gulosus</i>	2	0	114	Tyus 1973

<i>Ambloplites rupestris</i>	<i>Lepomis macrochirus</i> *	2	5	2460	Tyus 1973
<i>Ambloplites rupestris</i> *	<i>Lepomis macrochirus</i>	2	26	176	Tyus 1973
<i>Ambloplites rupestris</i>	<i>Micropterus salmoides</i> *	2	0	315-11,109	Hester 1970
<i>Ambloplites rupestris</i>	<i>Micropterus salmoides</i> *	2	0	n/a	Philipp et al. 1983
<i>Ambloplites rupestris</i>	<i>Micropterus salmoides</i> *	2	0	1796	Tyus 1973
<i>Ambloplites rupestris</i> *	<i>Micropterus salmoides</i>	2	0	n/a	Philipp et al. 1983
<i>Ambloplites rupestris</i> *	<i>Micropterus salmoides</i>	2	0	137	Tyus 1973
<i>Ambloplites rupestris</i>	<i>Pomoxis nigromaculatus</i> *	5	87	3174	Tyus 1973
<i>Ambloplites rupestris</i>	<i>Pomoxis nigromaculatus</i> *	5	86	315-11,109	Hester 1970
<i>Ambloplites rupestris</i> *	<i>Pomoxis nigromaculatus</i>	5	41	184	Tyus 1973
<i>Centrarchus macropterus</i>	<i>Lepomis macrochirus</i> *	2	12	315-11,109	Hester 1970
<i>Centrarchus macropterus</i>	<i>Micropterus floridanus</i> *	2	0	n/a	Parker et al. 1985b
<i>Enneacanthus gloriosus</i>	<i>Lepomis macrochirus</i> *	2	22	315-11,109	Hester 1970
<i>Enneacanthus gloriosus</i>	<i>Micropterus floridanus</i> *	2	10	n/a	Parker et al. 1985b
<i>Enneacanthus obesus</i>	<i>Micropterus floridanus</i> *	2	0	n/a	Parker et al. 1985b
<i>Lepomis auritus</i>	<i>Lepomis macrochirus</i> *	7	122	315-11,109	Hester 1970
<i>Lepomis auritus</i>	<i>Lepomis macrochirus</i> *	7	115	20	Smitherman and Hester 1962

<i>Lepomis auritus</i>	<i>Lepomis microlophus</i> *	10	64	315-11,109	Hester 1970
<i>Lepomis auritus</i>	<i>Lepomis microlophus</i> *	10	107	60	Smitherman and Hester 1962
<i>Lepomis cyanellus</i>	<i>Lepomis gulosus</i> *	9	107	276	Childers 1967
<i>Lepomis cyanellus</i> *	<i>Lepomis gulosus</i>	9	97	678	Childers 1967
<i>Lepomis cyanellus</i> *	<i>Lepomis gulosus</i>	9	101	300	Parker et al. 1985a
<i>Lepomis cyanellus</i>	<i>Lepomis macrochirus</i> *	8	79	315-11,109	Hester 1970
<i>Lepomis cyanellus</i>	<i>Lepomis macrochirus</i> *	8	100	639	Childers 1967
<i>Lepomis cyanellus</i> *	<i>Lepomis macrochirus</i>	8	140	103	Smitherman and Hester 1962
<i>Lepomis cyanellus</i> *	<i>Lepomis macrochirus</i>	8	91	589	Childers 1967
<i>Lepomis cyanellus</i> *	<i>Lepomis macrochirus</i>	8	85	300	Parker et al. 1985a
<i>Lepomis cyanellus</i> *	<i>Lepomis megalotis</i>	7	110	300	Parker et al. 1985a
<i>Lepomis cyanellus</i>	<i>Lepomis microlophus</i> *	7	118	552	Childers 1967
<i>Lepomis cyanellus</i>	<i>Lepomis microlophus</i> *	7	96	115	Smitherman and Hester 1962
<i>Lepomis cyanellus</i> *	<i>Lepomis microlophus</i>	7	111	597	Childers 1967
<i>Lepomis cyanellus</i> *	<i>Lepomis microlophus</i>	7	125	190	Smitherman and Hester 1962
<i>Lepomis cyanellus</i>	<i>Micropterus salmoides</i> *	3	80	n/a	Philipp et al. 1983
<i>Lepomis cyanellus</i> *	<i>Micropterus salmoides</i>	3	11	n/a	Philipp et al. 1983

<i>Lepomis gulosus</i>	<i>Lepomis macrochirus</i> *	8	25	315-11,109	Hester 1970
<i>Lepomis gulosus</i>	<i>Lepomis macrochirus</i> *	8	34	108	Smitherman and Hester 1962
<i>Lepomis gulosus</i>	<i>Lepomis macrochirus</i> *	8	70	699	Childers 1967
<i>Lepomis gulosus</i>	<i>Lepomis macrochirus</i> *	8	10	n/a	Merriner 1971
<i>Lepomis gulosus</i>	<i>Lepomis macrochirus</i> *	8	38	n/a	West and Hester 1966
<i>Lepomis gulosus</i> *	<i>Lepomis macrochirus</i>	8	64	315-11,109	Hester 1970
<i>Lepomis gulosus</i> *	<i>Lepomis macrochirus</i>	8	100	311	Childers 1967
<i>Lepomis gulosus</i> *	<i>Lepomis macrochirus</i>	8	86	n/a	Merriner 1971
<i>Lepomis gulosus</i> *	<i>Lepomis macrochirus</i>	8	58	n/a	West and Hester 1966
<i>Lepomis gulosus</i>	<i>Lepomis microlophus</i> *	7	82	548	Childers 1967
<i>Lepomis gulosus</i> *	<i>Lepomis microlophus</i>	7	107	317	Childers 1967
<i>Lepomis gulosus</i>	<i>Micropterus floridanus</i> *	3	70	n/a	Parker et al. 1985b
<i>Lepomis gulosus</i>	<i>Micropterus salmoides</i> *	3	104	315-11,109	Hester 1970
<i>Lepomis gulosus</i>	<i>Micropterus salmoides</i> *	3	97	n/a	West and Hester 1966
<i>Lepomis gulosus</i>	<i>Micropterus salmoides</i> *	3	116	n/a	Merriner 1971
<i>Lepomis gulosus</i>	<i>Micropterus salmoides</i> *	3	93	n/a	Philipp et al. 1983
<i>Lepomis gulosus</i> *	<i>Micropterus salmoides</i>	3	71	315-11,109	Hester 1970

<i>Lepomis gulosus</i> *	<i>Micropterus salmoides</i>	3	62	n/a	West and Hester 1966
<i>Lepomis gulosus</i> *	<i>Micropterus salmoides</i>	3	96	n/a	Merriner 1971
<i>Lepomis gulosus</i> *	<i>Micropterus salmoides</i>	3	72	n/a	Philipp et al. 1983
<i>Lepomis gulosus</i>	<i>Pomoxis nigromaculatus</i> *	2	2	315-11,109	Hester 1970
<i>Lepomis gulosus</i>	<i>Pomoxis nigromaculatus</i> *	2	0	n/a	West and Hester 1966
<i>Lepomis gulosus</i>	<i>Pomoxis nigromaculatus</i> *	2	5	n/a	Merriner 1971
<i>Lepomis gulosus</i> *	<i>Pomoxis nigromaculatus</i>	2	1	315-11,109	Hester 1970
<i>Lepomis gulosus</i> *	<i>Pomoxis nigromaculatus</i>	2	0	n/a	West and Hester 1966
<i>Lepomis gulosus</i> *	<i>Pomoxis nigromaculatus</i>	2	4	n/a	Merriner 1971
<i>Lepomis macrochirus</i>	<i>Lepomis microlophus</i> *	7	110	315-11,109	Hester 1970
<i>Lepomis macrochirus</i>	<i>Lepomis microlophus</i> *	7	91	1349	Smitherman and Hester 1962
<i>Lepomis macrochirus</i>	<i>Lepomis microlophus</i> *	7	105	512	Childers 1967
<i>Lepomis macrochirus</i> *	<i>Lepomis microlophus</i>	7	68	315-11,109	Hester 1970
<i>Lepomis macrochirus</i> *	<i>Lepomis microlophus</i>	7	63	2592	Smitherman and Hester 1962
<i>Lepomis macrochirus</i> *	<i>Lepomis microlophus</i>	7	99	742	Childers 1967
<i>Lepomis macrochirus</i>	<i>Micropterus floridanus</i> *	3	55	300	Parker et al. 1985a
<i>Lepomis macrochirus</i>	<i>Micropterus salmoides</i> *	3	66	315-11,109	Hester 1970

<i>Lepomis macrochirus</i>	<i>Micropterus salmoides</i> *	3	55	n/a	West and Hester 1966
<i>Lepomis macrochirus</i>	<i>Micropterus salmoides</i> *	3	83	n/a	Merriner 1971
<i>Lepomis macrochirus</i>	<i>Micropterus salmoides</i> *	3	77	n/a	Philipp et al. 1983
<i>Lepomis macrochirus</i> *	<i>Micropterus salmoides</i>	3	9	315-11,109	Hester 1970
<i>Lepomis macrochirus</i> *	<i>Micropterus salmoides</i>	3	9	n/a	West and Hester 1966
<i>Lepomis macrochirus</i> *	<i>Micropterus salmoides</i>	3	10	n/a	Merriner 1971
<i>Lepomis macrochirus</i> *	<i>Micropterus salmoides</i>	3	2	n/a	Philipp et al. 1983
<i>Lepomis macrochirus</i>	<i>Pomoxis nigromaculatus</i> *	2	39	315-11,109	Hester 1970
<i>Lepomis macrochirus</i>	<i>Pomoxis nigromaculatus</i> *	2	46	n/a	West and Hester 1966
<i>Lepomis macrochirus</i>	<i>Pomoxis nigromaculatus</i> *	2	48	n/a	Merriner 1971
<i>Lepomis macrochirus</i> *	<i>Pomoxis nigromaculatus</i>	2	11	315-11,109	Hester 1970
<i>Lepomis macrochirus</i> *	<i>Pomoxis nigromaculatus</i>	2	18	n/a	West and Hester 1966
<i>Lepomis macrochirus</i> *	<i>Pomoxis nigromaculatus</i>	2	10	n/a	Merriner 1971
<i>Lepomis microlophus</i>	<i>Micropterus floridanus</i> *	3	91	n/a	Parker et al. 1985b
<i>Lepomis microlophus</i>	<i>Micropterus salmoides</i> *	3	43	n/a	Philipp et al. 1983
<i>Lepomis microlophus</i> *	<i>Micropterus salmoides</i>	3	0	n/a	Philipp et al. 1983
<i>Lepomis minneatus</i>	<i>Micropterus floridanus</i> *	3	55	n/a	Parker et al. 1985b

<i>Micropterus dolomieu</i>	<i>Micropterus salmoides</i> *	11	102	n/a	Philipp et al. 1983
<i>Micropterus dolomieu</i> *	<i>Micropterus salmoides</i>	11	82	n/a	Philipp et al. 1983
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	97	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	69	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	80	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	82	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	95	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	97	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	112	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	91	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	91	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	89	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	90	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	94	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	96	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	83	100	Philipp et al. 1985
<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	94	100	Philipp et al. 1985

<i>Micropterus floridanus</i>	<i>Micropterus salmoides</i> *	12	93	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	115	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	96	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	121	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	117	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	108	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	116	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	107	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	115	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	98	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	98	100	Philipp et al. 1985
<i>Micropterus floridanus</i> *	<i>Micropterus salmoides</i>	12	109	n/a	Parker et al. 1985b
<i>Micropterus floridanus</i> *	<i>Pomoxis nigromaculatus</i>	2	1	n/a	Parker et al. 1985b
<i>Micropterus salmoides</i>	<i>Pomoxis annularis</i> *	2	0	n/a	Philipp et al. 1983
<i>Micropterus salmoides</i> *	<i>Pomoxis annularis</i>	2	0	n/a	Philipp et al. 1983
<i>Micropterus salmoides</i>	<i>Pomoxis nigromaculatus</i> *	2	0	315-11,109	Hester 1970
<i>Micropterus salmoides</i>	<i>Pomoxis nigromaculatus</i> *	2	0	n/a	West and Hester 1966

<i>Micropterus salmoides</i>	<i>Pomoxis nigromaculatus</i> *	2	0	n/a	Merriner 1971
<i>Micropterus salmoides</i> *	<i>Pomoxis nigromaculatus</i>	2	0	315-11,109	Hester 1970
<i>Micropterus salmoides</i> *	<i>Pomoxis nigromaculatus</i>	2	0	n/a	West and Hester 1966
<i>Micropterus salmoides</i> *	<i>Pomoxis nigromaculatus</i>	2	0	n/a	Merriner 1971
<i>Pomoxis annularis</i>	<i>Pomoxis nigromaculatus</i> *	6	77	n/a	Epifano and Phillip 1994
<i>Pomoxis annularis</i> *	<i>Pomoxis nigromaculatus</i>	6	98	n/a	Epifano and Phillip 1994

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