

## RESEARCH ARTICLES

# Geological Dates and Molecular Rates: Fish DNA Sheds Light on Time Dependency

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Knowledge of DNA evolution is central to our understanding of biological history, but how fast does DNA change? Previously, pedigree and ancient DNA studies—focusing on evolution in the short term—have yielded molecular rate estimates substantially faster than those based on deeper phylogenies. It has recently been suggested that short-term, elevated molecular rates decay exponentially over 1–2 Myr to long-term, phylogenetic rates, termed “time dependency of molecular rates.” This transition has potential to confound molecular inferences of demographic parameters and dating of many important evolutionary events. Here, we employ a novel approach—geologically dated changes in river drainages and isolation of fish populations—to document rates of mitochondrial DNA change over a range of temporal scales. This method utilizes precise spatiotemporal disruptions of linear freshwater systems and hence avoids many of the limitations associated with typical DNA calibration methods involving fossil data or island formation. Studies of freshwater-limited fishes across the South Island of New Zealand have revealed that genetic relationships reflect past, rather than present, drainage connections. Here, we use this link between drainage geology and genetics to calibrate rates of molecular evolution across nine events ranging in age from 0.007 Myr (Holocene) to 5.0 Myr (Pliocene). Molecular rates of change in galaxiid fishes from calibration points younger than 200 kyr were faster than those based on older calibration points. This study provides conclusive evidence of time dependency in molecular rates as it is based on a robust calibration system that was applied to closely related taxa, and analyzed using a consistent and rigorous methodology. The time dependency observed here appears short-lived relative to previous suggestions (1–2 Myr), which has bearing on the accuracy of molecular inferences drawn from processes operating within the Quaternary and mechanisms invoked to explain the decay of rates with time.

## Introduction

The “molecular clock” concept has underpinned recent advances in evolutionary biology, facilitating hypothesis testing and elucidating timescales of biodiversification (Arbogast et al. 2002; Bromham 2003; Bromham and Penny 2003). In parallel, use of coalescent theory has enabled molecular biologists to estimate demographic parameters such as effective population sizes and migration rates (Kuhner et al. 1995; Beerli and Felsenstein 1999; Hey 2005). But the accuracy of such studies depends on knowledge of underlying mutation rates. In the majority of cases, rates of DNA change have been determined by calibrating differences in DNA sequences against an estimate of lineage divergence time, based on independent fossil or paleobiogeographic data, typically of Tertiary age (1.5–65 Myr; e.g., Brown et al. 1979, 1982; supplementary table S1, Supplementary Material online). More recently, rates of DNA change have been estimated using precisely dated subfossil material, up to 60 kyr old (e.g., Lambert et al. 2002; Shapiro et al. 2004; Edwards et al. 2007). At the shallowest level, rates have been calibrated directly from the number of changes that have accumulated along pedigrees or mutation accumulation lines (e.g., Denver et al. 2000; Howell et al. 2003; Santos et al. 2005).

Although it has long been recognized that different taxa and genes experience different rates of DNA change (Arbogast et al. 2002; Bromham and Penny 2003; Gillooly

et al. 2005), recent studies have also suggested that rates decline with increasing evolutionary timescale: the so-called “time dependency of molecular rates” (Ho et al. 2005; Ho and Larson 2006) or “lazy J” curve (Penny 2005). With respect to the mitochondrial DNA (mtDNA) control region in humans, for example, pedigree rates are fastest (e.g., 0.51 mutations/site/Myr; Santos et al. 2005), followed by ancient DNA (aDNA) rates based on 10.3 kyr remains (0.34–0.44 mutations/site/Myr; Kemp et al. 2007), and then “phylogenetic” estimates derived from Neogene primate divergences (0.05–0.24 mutations/site/Myr; Santos et al. 2005; Emerson 2007). Although some of this discrepancy in rates may be explained by differences in methodologies and their errors (Bromham 2003; Bromham and Penny 2003; Howell et al. 2003; Santos et al. 2005), it appears that appropriate rates need to be applied not only with respect to taxon and gene, but also the timescale of the question at hand (Ho et al. 2005; Ho and Larson 2006). However, the time of transition between fast “pedigree-like” rates and asymptotic “deep-phylogeny” rates is under question (Ho et al. 2005; Emerson 2007; Ho, Shapiro, et al. 2007). Satisfactory resolution of this issue requires the application of a consistent calibration methodology across a time frame ranging from thousands to millions of years, in a group of closely related species. In the current study, therefore, we assess molecular evolutionary rates across a broad temporal scale (0.007–5.0 Myr), based on 9 geological events effecting isolation of freshwater fish populations, mostly within a recent radiation of galaxiid fishes (Waters and Wallis 2001; Waters and McDowall 2005).

New Zealand’s South Island (fig. 1) is a geologically dynamic and mountainous region that has experienced extensive glaciation and tectonic uplift during the last 5 Myr

Key words: molecular clock, mutation rate, calibration, time dependency, purifying selection.

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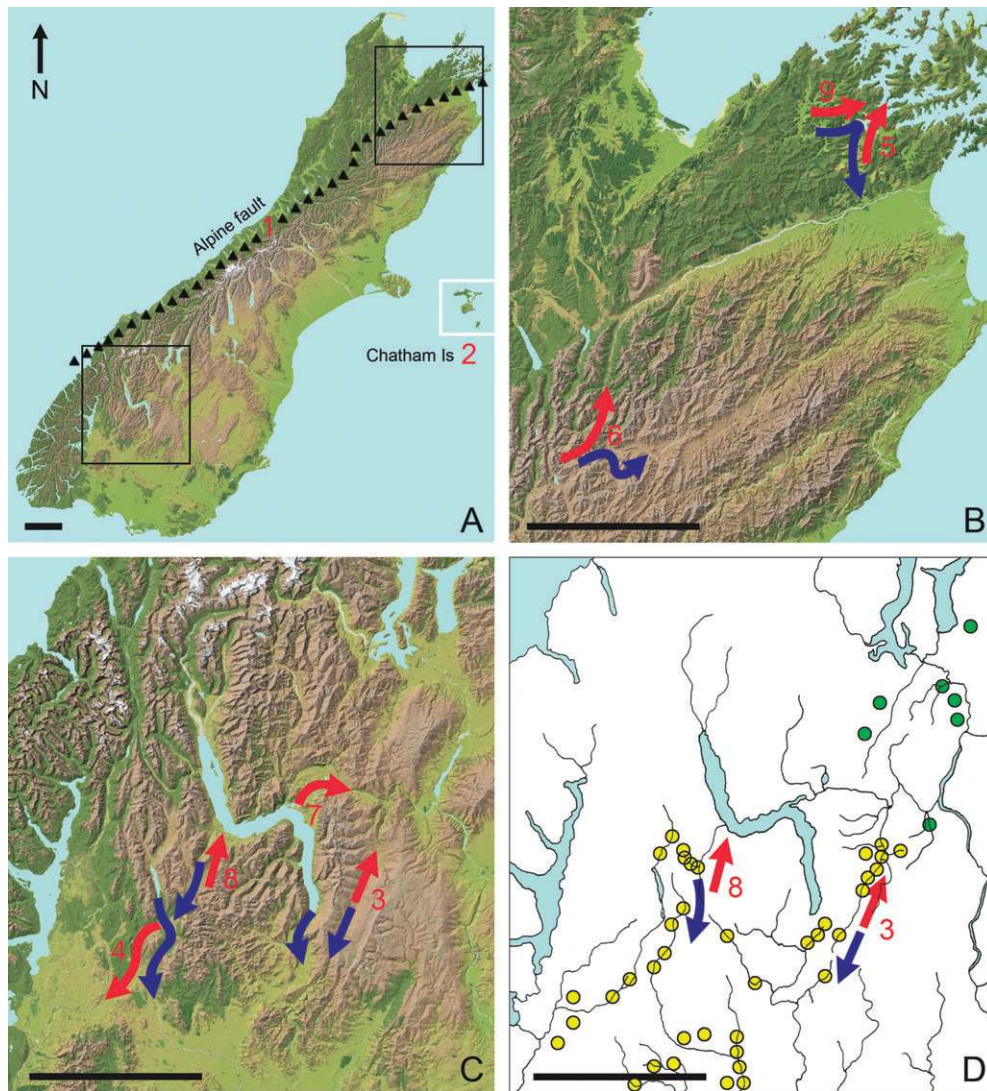


FIG. 1.—The study area. Numbers represent dated freshwater isolation events employed during calibration of molecular rates in New Zealand fishes (see table 1 for details). Blue arrows represent historical river directions, whereas red arrows represent contemporary directions. Scale bars are 50 km. (A) South Island, New Zealand, showing the Alpine Fault and eastern uplift of the Southern Alps. The Chatham Islands were formed approximately 800 km east of New Zealand. (B) Detailed view of the northeast South Island, showing river capture, reversal, and valley drowning events (numbers 6, 5, and 9, respectively). (C) Detailed view of the southwest South Island, showing river capture (numbers 4, 7, and 8) and reversal (number 3) events. (D) Examples of fish phylogeographic patterns reflecting historical rather than contemporary drainage connections. Nevis (3) and Von (8) rivers contain *Galaxias gollumoides* (yellow circles) rather than *Galaxias* sp. (green circles), reflecting the former southern connections of these drainages.

(Sutherland 1996). This isolated, active landscape presents a superb system for studying the effects of geological activity on biological evolution. Recent geological studies have revealed dynamic histories for many South Island river systems (fig. 1; e.g., McAlpin 1992; Mortimer and Wopereis 1997; Turnbull 2000; Craw and Norris 2003). These histories include a number of river reversal and river capture events (fig. 2)—driven by tectonics and glaciation—that involved wholesale transfer of streams between adjacent river catchments. Such river evolution effects vicariant isolation of freshwater-limited fish populations that were previously connected (fig. 2) and can lead to cladogenesis and the formation of new species (e.g., Waters and Wallis 2001; BurrIDGE et al. 2006; Waters et al. 2007). In addition, the extent of genetic divergence among popula-

tions is strongly correlated with their age of physical isolation, as determined using geological techniques—correlation of river terrace profiles with Oxygen Isotope glacial stages (Craw and Waters 2007). Our study uses this causal link between drainage geology and genetics to quantify rates of DNA change. This novel calibration method is based on precise spatiotemporal disruptions of linear freshwater systems and hence avoids many of the limitations associated with typical DNA calibration methods, where the link between a geological event (e.g., fossil deposition, island formation) and population isolation is not necessarily synchronous.

Here, we use 7 geologically dated river evolution events—ranging from 7,000 to 500,000 years old—to measure rates of mtDNA change in New Zealand

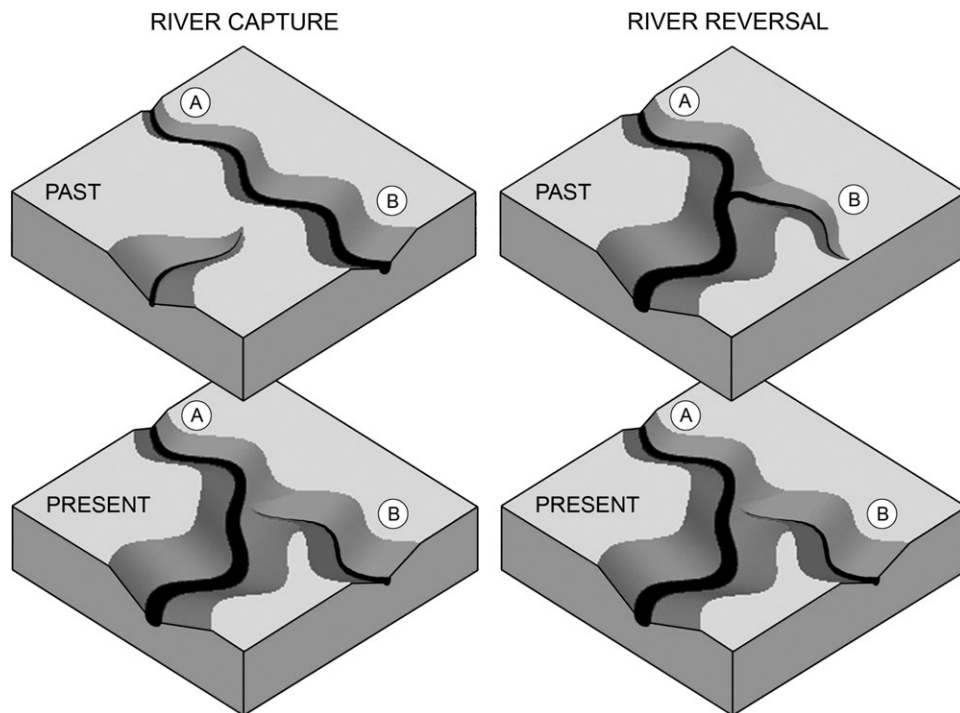


FIG. 2.—River capture and river reversal. Freshwater-limited populations located in stream sections “A” and “B” become genetically isolated following changes to river drainage geometry. “River capture” represents the displacement of stream sections between adjacent catchments, vicariantly isolating populations that previously inhabited the same system. Similarly, “river reversal” within part of a drainage will result in vicariant isolation of populations on either side of the new drainage divide, as will marine inundation of a stream junction (data not shown).

freshwater-limited fishes. In addition, we employ 2 older events: the Pliocene uplift of the Southern Alps and the mid-Pleistocene emergence of the Chatham Islands (fig. 1). In comparison to the river evolution events, these 2 older tectonic events lack robust upper and lower bounds of time since population isolation but in combination provide a deeper level contrast of mtDNA rates. In total, 13 independent estimates of molecular rates are calculated for galaxiid fishes (Galaxiidae) and 3 for an eleotrid fish species (Eleotridae), spanning 0.007–5.0 Myr (table 1). In each case, mitochondrial cytochrome *b* and control region sequences have been obtained from populations with known geological isolation histories, and a consistent coalescent methodology is employed to estimate associated rates of DNA change.

## Materials and Methods

### Genetic Analyses

Fish sampling (galaxiids, eleotrids) associated with each calibration point is summarized in table 1 and was approved by the University of Otago Animal Ethics Committee. At least 50 individuals were analyzed for 13 of the 16 independent calibrations performed. This does not include additional individuals sampled from other catchments in the region to confirm that genetic relationships were consistent with the hypothetical paleodrainage connections rather than some unexpected geological history. Sampling effort was geographically spread throughout the catchments that had undergone modification to their paleodrainage bound-

aries, such that the majority of genetic diversity would be encompassed. Intracatchment phylogeographic structuring is typically absent in these taxa (e.g., Burridge et al. 2006; Waters et al. 2007) and appears only in conjunction with large in-stream features such as gorges (Burridge et al. 2007), which are lacking within the systems we have employed for calibrations. Consequently, the geological age of isolation events should be applicable to the patterns of molecular variation observed. Laboratory protocols for the sequencing of mitochondrial cytochrome *b* gene and control region were as previously described (Waters et al. 2007). For galaxiids, cytochrome *b* sequences were at least 762 bp and control region 664 bp. Corresponding *Gobiomorphus breviceps* (Eleotridae) sequences were 900 and 385 bp. Potential heteroplasmies were scored as ambiguous nucleotides, which were subsequently ignored during coalescent estimation of rates. Sequence data are available from GenBank under accession numbers reported previously (Davey et al. 2003; Burridge et al. 2006, 2007; Waters et al. 2007) plus EU309048–469. Phylogeographic relationships were reconstructed via maximum parsimony, maximum likelihood, and Bayesian analysis and were concordant with the geological histories of river isolation predicted *a priori* (Waters and Wallis 2001; Burridge et al. 2006, 2007; Waters et al. 2007). An overview of phylogenetic relationships among galaxiids, and inferred geological isolation events, is given in supplementary figure S1, Supplementary Material online. For *Gobiomorphus breviceps*, Smith et al. (2005) detail the divergence populations on either side of the Southern Alps, and Waters et al. (2007) describe the relationships among the Pelorus, Kaituna, and Wairau rivers.

**Table 1**  
**Summary of Freshwater Isolation Events Employed for the Calibration of Mutation Rates**

Isolation Event (number in fig. 1)	Age (Myr), Mechanism	Species	Sample Sizes	Rate (changes/site/Myr)			Bayesian	dN:dS (2ΔlnL)	ω <sub>W</sub> /ω <sub>B</sub>	Geological References
				p distance	HKY distance	distance				
1. Southern Alps	2.0–5.0, mountain uplift	<i>Galaxias divergens</i> , <i>G. paucispindylus</i>	56, 42	0.0107	0.0117	0.0112	6.866*	405	Wellman (1979); Sutherland (1996)	
2. Chatham Is.	<1.0, island formation	<i>Gobiomorphus breviceps</i>	56, 20	0.0078	0.0081	0.0077	2.274	5	Campbell et al. (2006)	
3. Nevis–Mataura	0.300–0.500, river reversal	<i>Neochanna burrowsius</i> , <i>N. rekolua</i>	45, 24	0.0214	0.0231	0.0238	2.709	6	Waters and Wallis (2001); Youngson et al. (2002)	
4. Mararoa–Oreti	0.145–0.240, river capture	<i>Galaxias gollumoides</i>	42, 89	0.0199	0.0211	0.0258	5.463*	719	Turnbull (2000); Craw, Burridge, et al. (2007)	
		<i>Galaxias 'southern'</i>	54, 66	0.0523	0.0535	0.0235	5.585*	726		
		<i>G. paucispindylus</i>	42, 25	0.0090	0.0090	0.0150	1.596	897		
5. Kaituna–Wairau	0.070–0.130, river reversal	<i>G. divergens</i>	56, 39	0.0453	0.0459	0.0535	2.088	656	Lauder (1970); Mortimer and Wopereis (1997);	
		<i>G. breviceps</i>	38, 25	0.0360	0.0304	0.0248	0.141	634	Craw, Anderson, et al. (2007); Craw and Waters (2007)	
6. Clarence–Wairau	0.010–0.020, river capture	<i>G. divergens</i>	32, 41	0.0771	0.0825	0.1255	0.472	733	McAlpin (1992); Burridge et al. (2006);	
		<i>Galaxias 'northern'</i>	34, 31	0.0964	0.0805	0.0657	0.519	288	Rattenbury et al. (2006)	
		<i>G. paucispindylus</i>	5, 15	0.0173	0.0174	0.0310	<sup>a</sup>		Turnbull (2000)	
7. Lochy–Mataura	<0.020, river capture	<i>G. paucispindylus</i>	7, 23	<sup>b</sup>	<sup>b</sup>	0.0496	0.000	1	Turnbull (1980, 2000); Craw and Norris (2003)	
8. Von–Oreti	0.012, river capture	<i>G. 'southern'</i>	31, 66	<sup>b</sup>	<sup>b</sup>	0.0391	<sup>a</sup>		Gibb (1986); Singh (1994)	
		<i>G. gollumoides</i>	52, 66	0.1111	0.1141	0.0677	0.226	327		
		<i>G. divergens</i>	31, 25	0.0976	0.0991	0.0689	<sup>a</sup>			
9. Pelorus–Kaituna	0.007, valley drowning	<i>G. breviceps</i>	23, 15	0.1144	0.1140	0.1175	<sup>a</sup>			

<sup>a</sup> Test not possible due to polyphyletic relationships.

<sup>b</sup> Maximum intrapopulation divergence exceeds maximum interpopulation divergence.

\* $P < 0.05$ .

## Geological Analyses

The majority of calibration points were provided by changes in river drainage geometry (table 1), and the ages of such events were inferred by correlation of river terraces with previous glacial stages. River terraces are remnants of old river gravel deposits preserved on valley walls. These gravel deposits accumulated at times of high sediment supply in the catchments, typically during glaciations, and their gradients reflect paleodrainage direction. River terraces often occur as a series of “steps” up a valley wall, reflecting the past series of glaciations, and their relative ages can be determined over large areas by correlating sets of terraces as geomorphic features throughout river catchments. Absolute ages of terraces can be estimated by correlation with periods of global glaciation that have been determined with oxygen isotope ratios in the marine sedimentary record. A well-calibrated oxygen isotope timescale has been defined internationally (Chappell et al. 1996). Optically stimulated luminescence and radiocarbon dating of material from river terraces were employed to verify correlations with the international timescale. Three calibration dates were estimated from uplift rates and age of island emergence (table 1). Many of the paleodrainage events have ages constrained by both older (higher) and younger (lower) river terraces, thereby providing both objective and temporally accurate upper and lower bounds of population isolation, as opposed to events such as island colonization, where upper bounds may be erroneous (Emerson 2007) and lower bounds are subjective.

## Rate Calibration

Coalescent simulations under the 4-parameter isolation model (Wakeley and Hey 1997) were employed to estimate rates of DNA change using the IM software. Because the mitochondrial cytochrome *b* and control regions are linked during inheritance and their proportions of variable sites were similar (cytochrome *b* relative to control region: 0.73–2.24 in galaxiid data sets, 0.62–1.00 in *G. breviceps* data sets), they were combined for the estimation of a single mutation rate, to maximize the information content of the data. Nucleotide change was assumed to follow the Hasegawa-Kishino-Yano (HKY) model, which is the only model implemented by IM that can accommodate multiple substitutions at sites. This model appears adequate for the majority of the data sets, as HKY distances increase in a linear manner with those obtained under models selected from 56 candidates using a hierarchical likelihood ratio test (Posada and Crandall 1998)(supplementary fig. S2, Supplementary Material online). The nonlinear relationships observed from 3 of the older calibration data sets represent underestimation of molecular change at deeper divergence levels when using the HKY model, which could introduce slight error during the estimation of mutation rates.

Using the IM software, genealogical topologies were simulated and updated along a Markov chain Monte Carlo during which the 4 model parameters—divergence time (*t*), 2 contemporary and 1 ancestral population sizes ( $\theta$ )—were recorded. Where possible, upper bounds of parameter priors were set such that posterior distributions were fully

contained within them, with peaks representing maximum likelihood estimates. A minority of  $\theta$  posteriors contained infinite nonzero tails, but repeating analyses with different priors produced similar rate estimates. Markov chain Monte Carlo searches employed 4 chains, 3 of which were incrementally heated to promote broader searching of parameter space. After a burn-in period of  $10^5$  generations, parameter trendline plots were examined for the attainment of stationarity and parameter values were subsequently recorded every 10 generations. Independence of samples collected along a chain was assessed via their autocorrelation statistics, and runs were continued until the effective sample size exceeded 100 for all parameters. Independent runs using different random number seeds were conducted to assess convergence upon the true stationary distribution. Independence among parameter estimates was checked. Rates of DNA change ( $u$ ) were calculated based on geologically derived estimates of maximum generations elapsed since population isolation ( $t$ ), using the relationship  $t = \mathbf{t}u$ . We employed female generation times of 1 and 2 years for galaxiids and eleotrids, respectively (Hopkins 1971; Staples 1975; McDowall 2000; Hamilton and Poulin 2001).

Simple estimates of rates were also derived from net sequence distance,  $D_{\text{net}} = 0.5d_{xy} - 0.5\max\{d_x, d_y\}$ , where  $d$  is the sequence divergence either between ( $d_{xy}$ ) or within ( $d_x, d_y$ ) populations  $y$  and  $x$ . We employed both uncorrected ( $p$ ) distances and HKY distances. The latter provided comparisons for calibrations derived from the 4-parameter isolation model when the genealogies underlying the data sets were likely to be reciprocally monophyletic, as the time parameter ( $t$ ) may be strongly and negatively correlated with ancestral  $\theta$  under such conditions (Wakeley and Hey 1997).

### Purifying Selection

Tests of purifying selection were employed using the likelihood scores approach of Hasegawa et al. (1998). Likelihood scores were calculated for cytochrome *b* data sets using codeml of PAML 3.14 (Yang 1997) under either a single ratio of nonsynonymous to synonymous nucleotide changes (dN:dS) or 2 such ratios, with one constrained to population clades (e.g., catchments) and the other constrained to the branch linking population clades. Tree topologies and initial branch lengths were derived from Bayesian analysis of cytochrome *b* and control region data, but branch lengths were subsequently reestimated based on the cytochrome *b* data set and the M0 codon model (Yang et al. 2000). If purifying selection contributed to any time dependency of molecular rates, it is expected that the 2-ratio model will explain the data significantly better than the 1-ratio model as the interpopulation branch should have a lower dN:dS than the intrapopulation clades; this was assessed by likelihood ratio tests.

## Results and Discussion

### Time Dependency in Galaxiid and Eleotrid Fishes

The analysis of freshwater fish vicariance suggests a decline in mtDNA evolutionary rates with increasing age of calibration. Specifically, from coalescent analysis

we observe rates of 0.031–0.125 changes/site/Myr from river isolation events younger than 200 kyr in galaxiid fishes (table 1 and fig. 3). In contrast, galaxiid rates derived from older events are slower and less variable, in the order of 0.011–0.026 changes/site/Myr (table 1 and fig. 3), and compatible with most fish mtDNA rates based on older isolation events (supplementary table S1, Supplementary Material online). Confidence intervals of model parameters derived from weighted nonlinear least squares regression rejected a constant rate of molecular change with time (supplementary text S1, Supplementary Material online). The transition in coalescent-based rate estimates is also observed for rates derived from net sequence divergences (table 1 and fig. 3). These “simple” molecular rate estimates derived from  $p$  or HKY distances are broadly consistent with the coalescent rate estimates, but the larger discrepancies that exist may reflect inappropriate correction for sequence divergence present at the time of population isolation when using maximum contemporary intrapopulation divergence as a proxy. Strong negative correlations between the time parameter ( $t$ ) and ancestral  $\theta$  are expected during coalescent analysis of data sets likely to represent reciprocally monophyletic genealogies (Wakeley and Hey 1997), which could bias calibrations based on older isolation events. However, such correlations were positive or only slightly negative (e.g.,  $-0.12$ ) for the majority of the reciprocally monophyletic fish data sets, and rate estimates were similar to simple estimates derived under the same mutation model (HKY; table 1 and fig. 3).

The time-dependent transition observed here cannot be explained by interspecific rate variation (Gillooly et al. 2005); the calibrations are based on a closely related assemblage of fishes (supplementary fig. S1, Supplementary Material online; Galaxiidae: *Galaxias* and *Neochanna* spp.; Waters and Wallis 2001; Waters and McDowall 2005), and 7 of 8 transitions from a younger calibration event to the next oldest calibration within the same species of galaxiid were accompanied by a decline in molecular rate (fig. 3); the exception was the transition from 7 to 20 kyr calibrations for *Galaxias divergens*. “Time dependency” of a similar magnitude was also observed for a completely unrelated taxon, an eleotrid fish (*G. breviceps*), confirming that this pattern is not peculiar to the Galaxiidae (fig. 3).

Sequencing errors in the order of 1 in 1,000 bases (Wesche et al. 2004) could explain some of the elevated molecular rates we observed from calibrations of 20 kyr or younger, assuming that the true molecular rate in these fishes approximates the asymptotic estimate obtained here for galaxiids (0.01876 changes/site/Myr; supplementary text S1, Supplementary Material online). However, it should be reiterated that the rates presented here were derived from maximum ages of population isolation and hence are likely to have been underestimated, perhaps substantially. In addition, much higher sequencing error rates are required to explain elevated molecular rates obtained from older calibrations (see also Ho et al. 2005). Sequencing errors are also likely to be substantially less influential than aDNA damage, which has recently been refuted as a major contributor to elevated molecular rates observed from calibrations across short timescales (Ho, Heupink, et al. 2007).

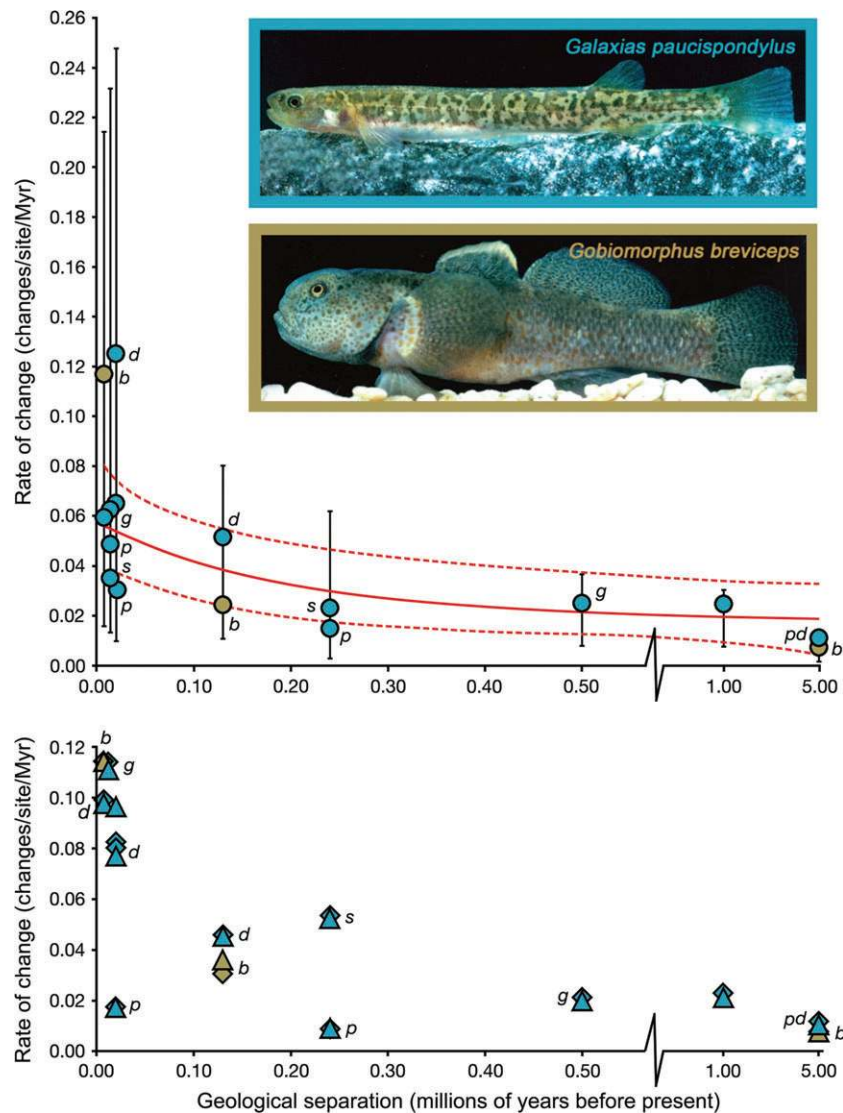


FIG. 3.—Molecular clock calibrations (nucleotide changes/site/Myr) derived from dated isolation events of New Zealand freshwater fish populations. Blue symbols represent galaxiid divergences (Galaxiidae), and brown symbols represent *Gobiomorphus breviceps* (Eleotriidae). Letters indicate rates derived from multiple events for the same species (g, *Galaxias gollumoides*; d, *Galaxias divergens*; s, *Galaxias 'southern'*; p, *Galaxias paucispondylus*; b, *Gobiomorphus breviceps*). Where ages are represented by both minimum and maximum estimates (table 1), we employed the latter, yielding minimum estimates of rates. The top graph represents coalescent-based estimates (error bars are the 90% highest posterior density). The red lines are vertically translated exponential decay curves for galaxiid data representing best-fit estimates (solid line,  $y = 0.01876 + 0.03911 \times e^{-5.25878x}$ ) and upper and lower 95% confidence intervals of model parameters (supplementary text S1, Supplementary Material online). The bottom graph represents “simple” rates based on net sequence divergences under either uncorrected (p distance, triangles) or HKY (diamonds) models of nucleotide substitution. Two young calibration events yielded negative simple rates (maximum intracatchment divergence exceeded intercatchment divergence, table 1) and are not shown on the lower graph.

Finally, errors in estimated ages of population isolation are unlikely to be a factor in our observation of declining molecular rates with time, given the temporal constraints provided by geological information. We therefore conclude that time dependency is the best explanation for the calibration data presented here.

#### The Duration of Time Dependency

Theoretical (Penny 2005) and some empirical (Howell et al. 2003; Santos et al. 2005) evidence for time dependency is strong, but determining the point at which

short-term (e.g., pedigree, aDNA) molecular rates decay to long-term (i.e., deep-phylogenetic) levels is crucial for genetic estimates of evolutionary timescales and demographic parameters (Ho et al. 2005; Ho and Larson 2006; Ho, Shapiro, et al. 2007). In galaxiid fishes, we observe “deep-phylogenetic” mtDNA rates (0.011–0.026 changes/site/Myr) beyond 200 kyr (fig. 3). Phylogenetic rates may also be attained earlier than 200 kyr, given the larger uncertainty surrounding rates from progressively younger calibration points. Therefore, our study suggests that elevated mtDNA rates might not persist much beyond 200 kyr in galaxiid fishes.

Attainment of asymptotic or deep-phylogenetic mtDNA rates at  $\approx 200$  kyr in galaxiid fishes (see above) would contrast with time dependent curves proposed for birds and mammals. Specifically, Ho et al. (2005) suggested that the duration of time dependency in mtDNA extended up to 2 Myr for primate protein-coding genes and up to 1 Myr for primate control region and avian protein-coding genes. While the duration of time dependency may vary among taxa, reflecting differences in generation time, effective population size, instantaneous mutation rate, and selection, a reanalysis of the Ho et al. (2005) data by Emerson (2007) provided no evidence of elevated rates for events older than 100 kyr, more consistent with our suggestions for galaxiid fishes of  $\approx 200$  kyr. Although a recent analysis of cichlid fish mtDNA control region sequences (Genner et al. 2007) suggested a pattern similar to that proposed by Ho et al. (2005), with attainment of asymptotic molecular rates after  $\approx 1$  Myr, there were no calibrations older than 50 kyr until 0.7 Myr, and the age of this and the next oldest calibration point were inferred from a molecular clock rather than directly from geological data. Therefore, an earlier attainment of baseline rates seems plausible for cichlids.

More recently, Ho, Shapiro, et al. (2007) argued for elevated mtDNA evolutionary rates in birds at scales greater than 100 kyr. However, their rock partridge rate of 0.125 substitutions/site/Myr—derived from a 238-kyr calibration point—is actually a lineage specific rate of 0.062 substitutions/site/Myr based on a 2-Myr isolation event, whose age was itself inferred from a molecular clock (Randi et al. 2003). Ho, Shapiro, et al. (2007) additional calibrations beyond 100 kyr yielding elevated rates are based on island emergence events and limited sampling (maximum 3 individuals per island), which could lead to overestimates of rates for several reasons, such as lineages predating island emergence or extinction of the closest mainland relative (Emerson 2007). Some elevated rates from events within the last 100 kyr may also require reinterpretation. For example, the *Bison* aDNA (0–60 kyr) rates reported by Ho, Shapiro, et al. (2007) are consistent with a phylogenetic rate derived from *Bison–Bos* divergence 1 Myr before present (Troy et al. 2001; see also Shapiro et al. 2004; Edwards et al. 2007). Hence, the duration of time dependency in birds, mammals, and cichlid fishes may match our suggestion for galaxiid fishes ( $\approx 200$  kyr), or in all cases, could be much more recent. Further work on this subject is required, employing robust calibration points.

### Mechanisms of Time Dependency

The suggestion of a relatively rapid ( $< 200$  kyr) attainment of asymptotic (deep-phylogenetic) rates in fishes from this study, which may also apply to birds, mammals, and cichlid fishes (see above), has implications for the mechanistic cause of time dependency. Recent studies have suggested that slightly deleterious mtDNA mutations contribute to rapid divergences across shallow timescales prior to their removal by purifying selection (Howell et al. 2003; Ho et al. 2005; Penny 2005; Santos et al. 2005), which is consistent with observations of higher ratios of nonsynonymous to synonymous nucleotide changes (dN:dS) at shallow versus deep phylogenetic levels (Hasegawa et al. 1998;

Ho et al. 2005; BurrIDGE et al. 2006; Kivisild et al. 2006; Rocha et al. 2006). Although recent simulations suggested that purifying selection alone could only explain time dependency if there were many significantly deleterious mutations and effective population sizes were implausibly large (Woodhams 2006), these results hinged on the decay curves reported by Ho et al. (2005). But if elevated rates do not persist much beyond 100 kyr, as we contend, time dependency might be readily explained by purification selection under plausible population sizes.

Analyses of dN:dS revealed significant signatures of purifying selection for 3 of the galaxiid data sets, representing 5 Myr, 0.5 Myr, and 0.24-Myr isolation events (table 1). Even in the nonsignificant data sets, dN:dS within catchments ( $\omega_W$ ) was greater than that along the branch between catchments ( $\omega_B$ , table 1). Consequently, the suggestion of purifying selection is quite persuasive, given the limited amount of nonsynonymous variation present in our data sets, reducing statistical power. At this stage, however, contributions by some alternative mechanisms cannot be discounted; these involve drift and random fluctuations in population size (Zihivovsky et al. 2006) or selective sweeps via linkage to sites experiencing positive selection, as recently proposed for mtDNA (Bazin et al. 2006). None of the above mechanisms are mutually exclusive.

Mutational hot spots—sites that undergo molecular change at very short time intervals but quickly become saturated such that the apparent accumulation of change decreases with time—have previously been invoked for observations of elevated rates across short timescales (Pääbo 1996; Jazin et al. 1998). However, it does not appear that the decline in fish molecular rates with time observed herein relates predominantly to mutational hot spots. We have assessed the location of variable sites and the numbers of nucleotide states at variable sites, among closely related clades of galaxiids. However, there is no similarity or clustering in the locations of intraclade polymorphisms in either cytochrome *b* or control region, and very few polymorphic sites exhibit more than 2 nt states, as would be anticipated if rapid molecular divergence across short timescales was the result of mutational hot spots (BurrIDGE et al. 2006). However, mutational hot spots appear to make contributions to elevated rates in other data sets, where regions of repeated nucleotide sequences exist (Denver et al. 2000), although in that instance, as we suggest in ours, the presence of mutational hot spots does not appear to be a prerequisite for the observation of elevated rates across short timescales.

### Implications of Time Dependency in Fishes

A major implication of time dependency is that estimates of population divergence times and demographic parameters may require reestimation (Ho et al. 2005; Ho and Larson 2006). For example, female effective population size ( $N_{ef}$ ) in shortnose sturgeon (*Acipenser brevirostrum*), derived from a phylogenetic mtDNA control region calibration of 0.018 changes/site/Myr, exceeded a census population estimate by over an order of magnitude (Quattro et al. 2002), whereas the reverse relationship is expected (Turner et al. 2006). In contrast, a calibration of

0.109–0.131 changes/site/Myr—derived from a postglacial (10–12 kyr) colonization event in white sturgeon (*Acipenser transmontanus*)—yielded  $N_{ef}$  for the endangered Chinese sturgeon (*Acipenser sinensis*) consistent with observational estimates of effective size (Zhang et al. 2003). The white sturgeon calibration also produced molecular estimates of east Atlantic colonization by *Acipenser oxyrinchus* that closely matched a Holocene first appearance in the archaeological record (Ludwig et al. 2002), whereas a phylogenetic rate of 0.018 would have exceeded this by 10 kyr. Similarly, many studies have invoked previously unexpected glacial refugia to accommodate molecular estimates of population divergence time that predate the last glacial maximum (Weiss et al. 2000; Gum et al. 2005; Culling et al. 2006). However, the time-dependent rates observed here will convert such molecular divergences into inferences of post-Pleistocene population isolation. This problem may be particularly important for studies using genetic divergences to formulate predictions of biological response to future climate change.

### Summary

This study has conclusively demonstrated the presence of time dependency in rates of molecular change for mtDNA regions in freshwater-limited fishes. The duration of time dependency in these fishes appears shorter than some suggestions for other taxa (Ho et al. 2005; Genner et al. 2007; Ho, Shapiro, et al. 2007), but we contend that shorter durations may apply to these groups as well. Under such a scenario, time dependency may be more readily explained by purifying selection than previously inferred (Woodhams 2006), although other mechanisms are also likely to contribute (drift and random fluctuations in population size, positive selection, and mutational hot spots associated with repeated nucleotide sequences). However, a shorter duration of time dependency does not remove the need for a careful reassessment of published estimates of population divergence times and demographic parameters derived from deep-phylogenetic molecular rate calibrations. However, the news is not all bad; using established molecular techniques, but with an appreciation of time dependency, we can now address important evolutionary questions that were previously considered too recent for existing approaches.

### Supplementary Material

Supplementary table S1, figures S1 and S2, and text S1 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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