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Playing chicken (*Gallus gallus*): methodological inconsistencies of molecular divergence date estimates due to secondary calibration points

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Abstract

For any given taxonomic divergence event, one may find in the literature a wide range of time estimates. Many factors contribute to the variation in molecular date estimates for the same evolutionary event. High on the list is the choice of calibration points for converting genetic distances into evolutionary rates and, subsequently, into dates of divergence. In this study, we investigate one critical source of error in estimating divergence times, i.e. the use of secondary calibration points, which are divergence time estimates that have been derived from one molecular dataset on the basis of a primary external calibration point, and which are used again independently of the original external calibration point on a second dataset. Unless particular care is exercised, this practice leads to internal inconsistencies, and the inferred dates of divergence are by necessity unreliable. We present a consistency test for assessing the reliability of divergence time estimates based on secondary calibration points. As a case study, we examine recent estimates of divergence times among phyla and kingdoms based on multiple nuclear protein-coding genes, and show that they fail the consistency test.

Keywords: Molecular date estimates; Divergence time; Calibration points; Consistency test

1. Introduction

The prevailing consensus among molecular biologists is that most taxa had diverged phylogenetically from one another long before they diversified morphologically. The question 'how long before?' though, is currently subject to considerable disagreement (Benton, 1999; Easteal, 1999). For any given divergence event, one may find in the literature a wide range of time estimates. Many factors contribute to the variation in molecular date estimates for the same evolutionary event. High on the list are: (1) different molecular datasets; (2) different criteria for inclusion or exclusion of data; (3) different methodologies for the derivation of genetic distances; and (4) different calibration points for converting genetic distances into evolutionary rates and subsequently into dates of divergence (for discussion, see Easteal, 1999; Wang et al., 1999; Bromham et al., 2000).

In this study, we would like to draw attention to errors arising from a particular type of methodological inconsistency, i.e. the use of secondary (or indirect) calibration points. A secondary calibration point is a divergence time estimate which has been derived from one molecular dataset on the basis of a primary external calibration point – usually one based on paleontological considerations – and which is used again independently of the original external calibration point on a second dataset.

As a case study, we shall examine the data in Wang et al. (1999), who used a secondary divergence time estimate of 110 MYA for the rodent–primate split (Hedges et al., 1996; Kumar and Hedges, 1998), whenever the lack of homologous avian sequences prevented them from using the primary paleontological estimate of 310 MYA for the bird–mammal divergence event. We note that secondary time estimates are used quite frequently for purposes of calibration (e.g. Gu, 1998; Heckman et al., 2001; Hedges et al., 2001).

2. Data and methods

2.1. Calibration dates

Following Wang et al. (1999), we shall use a bird–mammal divergence time of 310 MYA as the primary

Abbreviations: MYA, million years ago; T_1, time of divergence between primates and rodents; T_2, time of divergence between birds and mammals.

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calibration, and a rodent–primate estimate of 110 MYA as the secondary calibration. We note, however, that values for the secondary calibration point exhibit some variation in the literature, from 95 to 112 MYA (Hedges et al., 1996; Kumar and Hedges, 1998; Easteal, 1999). This variation can only be partly explained by the use of different datasets or by relaxation of criteria for inclusion within the same dataset. We also note that the primary calibration date is by no means universally accepted. For example, in our case study, the divergence between birds and mammals is based on paleontological evidence concerning the divergence between synapsids (to which mammals belong) and diapsids (to which birds belong). However, there exists no universal agreement among paleontologists on the 310 MYA date (e.g. Lee, 1999). Indeed, even the placement of synapsids as a sister taxon of the diapsids has been questioned (Kumazawa and Nishida, 1999).

2.2. Molecular data

The Wang et al. (1999) data contain 75 sets of homologous proteins. Seventy-four sets contain sequences from both primates and rodents, but only 29 sets contain an avian sequence (always *Gallus gallus*). Thus, most sets of proteins in Wang et al. (1999) lacked a primary calibration point, and in absentia a secondary one was used. In the following, we check the appropriateness of using secondary calibration points by subjecting the results to a consistency test.

2.3. Number of amino acid replacements between two proteins

The numbers of amino acid replacements between two aligned proteins were calculated with the Poisson correction. We note that the results remain essentially unchanged when more sophisticated methods of estimation are used (e.g. Ota and Nei, 1994).

In this note, outliers are treated in a more rigorous manner than in Wang et al. (1999). That is, instead of deciding a priori that two, four, or six extreme values must be thrown out from each dataset, we use Grubb's extreme studentized deviate test (Barnett and Lewis, 1994) in an iterative manner to identify statistically significant outliers.

2.4. Consistency test

We denote by T_1 the time of divergence between primates and rodents, and by T_2 the divergence time between birds and mammals. The consistency test will employ the 29 sets of homologous proteins for which both mammal and avian sequences are available in Wang et al. (1999). To calculate T_1, we shall use the rate of amino acid replacement as inferred from the bird–mammal comparison by assuming a divergence time of 310 MYA.

$$T_1 = \frac{310 \times d_{PR}}{d_{BM}}$$

where d_{PR} is the number of amino acid replacements per site between primate and rodent, and d_{BM} is the number of amino acid replacements per site between bird and mammal. d_{BM} has been calculated as $(d_{BR} + d_{BP})/2$, where d_{BR} and d_{BP} are the numbers of amino acid replacements per site between bird and primate and between bird and rodent, respectively.

Similarly, to calculate T_2, we shall use the rate of amino acid replacement as inferred from the primate–rodent comparison by assuming a divergence time of 110 MYA.

$$T_2 = \frac{110 \times d_{BM}}{d_{PR}}$$

For a set of homologous proteins to pass the consistency

Table 1
Consistency test for 29 homologous protein datasets for which primate, rodent, and bird sequences are available[a]

Protein	T_1 (MYA)	T_2 (MYA)	$T1 < T2$
Aldehyde dehydrogenase	219	156	−
Aldolase	67	507	+
Alkaline phosphatase	104	328	+
α-Actinin[b]	272	125	−
Amidophosphoribosyltransferase	105	326	+
Aminolevulinate synthase	200	170	−
Aspartate aminotransferase	134	254	+
Dihydrofolate reductase	115	296	+
Disulfide isomerase	114	298	+
DNA polymerase γ	127	268	+
Enolase	229	149	−
Ferritin heavy chain	181	188	+
Fructose-2,6-bisphosphatase	66	513	+
Furin	81	419	+
Glutamate dehydrogenase	42	803	+
Glutamine synthetase	186	183	−
Glyceraldehyde 3-phosphate dehydrogenase	223	153	−
Lactate dehydrogenase	120	285	+
Na-K ATPase α chain	129	265	+
Na-K ATPase β chain	15	2333[c]	+
P53	103	331	+
P65	52	653	+
Phosphoenolpyruvate carboxykinase	167	204	+
Phosphoglycerate kinase	56	604	+
Pyruvate kinase	70	486	+
Transcription factor *Eryf1*	51	662	+
Transglutinamase	113	301	+
Triosephosphate isomerase	132	258	+
Tryptophan hydroxylase	186	184	−

[a] Data from Supplementary Information in Wang et al. (1999).
[b] The Supplementary Information in Wang et al. (1999) does not list an entry for human α-actinin. We chose without prejudice the protein with Accession number AAC17470.
[c] Outlier identified by Grubb's extreme studentized deviate test (Barnett and Lewis, 1994).

test, two conditions must be met: (1) $T_1 < T_2$, i.e. the divergence of birds and mammals predated the divergence between primates and rodents; and (2) the mean inferred $T_2 \approx 310$ MYA, i.e. by using the secondary calibration point we recover a divergence time estimate that is close to the primary paleontological estimate for the bird–mammal divergence. If these two conditions are not met, then we shall conclude that the use of the secondary calibration point is unjustified.

3. Results and discussion

The results of the consistency test are shown in Table 1. For seven homologous protein sets (24% of the data), we obtain $T_1 > T_2$, i.e. they fail the first part of the consistency test. Ominously, a quarter of the gene set suggests an earlier divergence time between rodents and primates than between Synapsida and Diapsida. Thus, questions of data authenticity, homoplasious evolution, and orthology assessment must be raised. Fortunately, since we are only interested in consistency rather than absolute estimates of times of divergence, and since for the second part we disregard these seven genes, such factors are not expected to affect our conclusions at all.

Of the remaining 22 sets, one set (Na-K ATPase β chain) yields an extreme outlier value for T_2 (Grubb's extreme studentized deviate test; Barnett and Lewis, 1994), and was therefore removed from further consideration. The mean T_2 for the remaining 21 proteins was 393 MYA with a 95% confidence interval of 315–471 MYA. Thus, the second condition of the consistency test is also violated, i.e. the mean inferred T_2 is significantly different from the primary calibration estimate of 310 MYA ($t = 2.21$, $P < 0.05$). We must, therefore, conclude that the use of secondary calibration points is unjustified.

We recognize that our results may be influenced by the variance of the time estimates, which may be very large (e.g. Ayala et al., 1998). Indeed, if the variance of T_2 were much larger, we could not have rejected the null hypothesis. However, the use of secondary calibration points illustrates a much broader problem in molecular time estimation studies, i.e. the lack of appropriate calibration points (e.g. Ayala et al., 1998).

Derivation of divergence dates from molecular data is a complicated proposition even at the best of times (Lee, 1999), and using secondary calibration times complicates matters unnecessarily. As an extreme measure, we would suggest not to derive divergence dates from molecular data at all. However, if one insists on turning sequences into time units, we would recommend (1) using multiple primary calibration points, thereby decreasing the reliance on a single point, (2) employing methodologies that can accommodate rate heterogeneity among taxa, and (3) presenting confidence intervals allowing explicit hypothesis testing of divergence times (e.g. Sanderson, 1997; Rambaut and Bromham, 1998).

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