

Letter to the Editor

Estimation of Nucleotide Substitution Rates in Eurotiomycete Fungi

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In the entire fungal kingdom, only DNA substitution rates in the *SSU rRNA* gene (Berbee and Taylor 1993, 2001) and amino acid substitution rates (Heckman et al. 2001) have been estimated and used to date fungal divergences. However, these molecules are not sufficiently variable to date events at or below the genus level. DNA sequences of protein-coding genes and the internal transcribed spacer (*ITS*) region are sufficiently variable, but their substitution rates are not known. In this article, we investigated the DNA substitution rates in the protein-coding genes and the *ITS* region by comparison with the *SSU rDNA* divergence in a representative fungal lineage, Eurotiomycetes (=plectomycetes) (Eriksson and Winka 1998). The Eurotiomycete lineage is a monophyletic class of Ascomycota (Berbee and Taylor 1992) and includes many economically important fungi, such as *Penicillium chrysogenum* (antibiotic production) and *Aspergillus oryzae* (soy sauce production), as well as many human pathogens such as *Coccidioides immitis* (lung disease), *Histoplasma capsulatum* (lung disease), and *Trichophyton rubrum* (athlete's foot). Estimating DNA substitution rates in quickly evolving molecules such as the *ITS* and protein-coding genes will not only provide a means to estimate the divergence times between lineages at and below the genus level, but in conjunction with coalescent theory, it may provide information for estimating epidemiological parameters such as effective population size (Watterson 1975) and recombination rates (Hey and Wakeley 1997).

With the aid of published Eurotiomycete phylogenies based on *SSU rDNA* (Ogawa, Yoshimura, and Sugiyama 1997; Sugiyama, Ohara, and Mikawa 1999), we searched for pairs of closely related species which had not only *SSU rDNA* sequences but also sequences of homologous protein-coding genes. Next, we compared the extent of differentiation at synonymous sites of protein-coding genes between sister taxa. It was found that saturation at synonymous sites of protein-coding loci is attained before divergence of the *SSU rDNA* sequences reaches 1%–2%. A 1%–2% divergence usually corresponds to divergences seen within a genus or among closely related genera. Therefore, the protein genes should be well suited to dating divergences at and below the genus level.

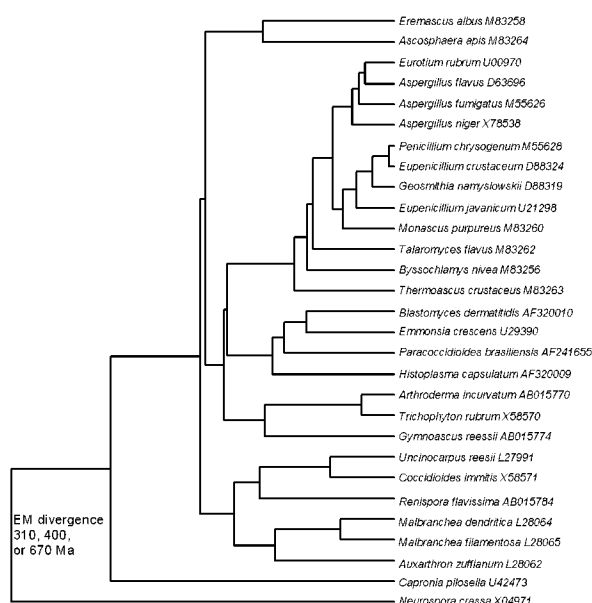
Key words: synonymous substitution rates, molecular clock, plectomycete, fungi, evolution, *ITS*.

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In this paragraph we briefly outline our methodology. The first step of our approach was to estimate the divergence times between sister species from the *SSU rDNA* phylogenetic tree (Ochman and Wilson 1987; Ochman, Elwyn, and Moran 1999). Twenty-seven Eurotiomycete species and two out-group taxa, i.e., the Sordariomycete *Neurospora crassa* and the loculoascomycete *Capronia pilosella*, were subjected to distance analysis. The regularity of nucleotide substitution rates at the *SSU rDNA* gene was evaluated by the two-cluster test and the branch length test (Takezaki, Rzhetsky, and Nei 1995; Nei and Kumar 2000). Rates were not found to be constant for six species and four nodes using the branch length test and the two-cluster test, respectively ($P < 0.05$, data not shown). Apparently, rate constancy does not hold throughout this system. Because rates may not be constant, we also considered a model in which molecular evolutionary rates vary across lineages instead of remaining constant. We compared the Langley Fitch (LF) algorithm, which assumes rate constancy, with the nonparametric rate-smoothing (NPRS) algorithm, which does not (Langley and Fitch 1974; Sanderson 1997). All methods of phylogenetic inference depend heavily on their underlying models. For this reason we used a hierarchical likelihood ratio test to search for the DNA substitution model that best fit our *SSU rDNA* data set (Posada and Crandall 1998). Phylogenetic relationships were then inferred using the selected evolutionary model and the heuristic search option for maximum likelihood (ML) implemented in PAUP 4.0b8a (Swofford 2001). The ML tree obtained was then used to estimate the divergence times between closely related taxa using the NPRS and LF methods. A simple and widely used Kimura two-parameter model was also used to construct a neighbor-joining (NJ) tree; the NJ tree was also subjected to the NPRS and LF methods as a comparison. Both NPRS and LF methods require at least one calibration point to fit branch lengths to the geological time scale. We used the divergence of Eurotiomycetes (=plectomycetes) and Sordariomycetes (=pyrenomycetes) for calibration. Initially, the divergence was set to 100 arbitrary units, and the relative divergence times between taxa were estimated (fig. 1; details are available in Supplementary Material at <http://www.molbioevol.org/>). For all pairs of taxa, the NPRS algorithm gave 15.2% to 87.5% larger values for divergence times than did the LF algorithm. For both NPRS and LF algorithms, most of the divergence values calculated from the NJ tree with a Kimura two-parameter algorithm were larger than those from the ML tree; NJ values were up to 48.6% larger than ML values. A simulation study indicated that divergence times are estimated more accurately using NPRS rather than LF when (1) there are enough data, (2) evolution is not clock-

a) NPRS



b) LF

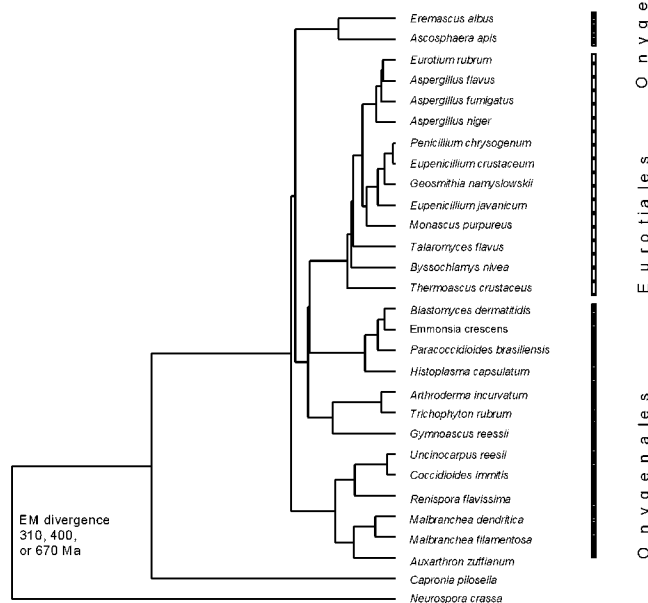


FIG. 1.—Estimation of divergence times according to the NPRS (a) and LF (b) methods. Branch lengths are proportional to time. Either of the three published values (310, 400, or 670 Myr) for the ES divergence time can be used to calibrate the trees. The tree topology was obtained through the heuristic search for ML implemented in PAUP 4.0b8a (Swofford 2001) using the Tamura Nei DNA substitution model with equal base frequencies, among-site rate variation Gamma (0.6431), and the proportion of sites unable to accept substitutions (0.6425). Aligned sequences have been deposited in TreeBase (study accession number S804, www.treebase.org/treebase/).

like, and (3) levels of rate autocorrelation are moderate to high (Sanderson 1997). Unfortunately, although we believe we have sufficient data (1,631 bp), we have no way of assessing the other parameters, so we have used the divergence times of both the NPRS and the LF methods, hoping to account for the ambiguity of the past.

As mentioned above, the NPRS and LF algorithms give only relative divergence times, which may be understood as a percentage of the time since the divergence of Eurotiomycetes and Sordariomycetes (ES). This ES divergence time can be inferred from the fossil record or estimated by molecular clock approaches, both of which again require calibration points from fossils. The oldest well-documented ascomycete fossils are the perithecia, asci, and ascospores that resemble those of extant Sordariomycetes and are found in the 400-Myr-old Rhynie chert (Taylor, Hass, and Kerp 1999). Thus, 400 Myr for the ES divergence would seem to provide a conservative date; however, earlier dates can be expected. The ES divergence time estimated from a molecular clock approach largely depends on a fundamental calibration point: the fungi-animal or fungi-animal-plant divergence time. Berbee and Taylor (Berbee and Taylor 2001) calibrated an *SSU rDNA* phylogenetic tree using a fungi-animal divergence time of 965 Myr (Doolittle et al. 1996) and estimated the ES divergence to be 310 Myr. Heckman et al. (2001) used a fungi-animal-plant divergence time of 1,576 Myr, and their protein diversity analyses yielded an ES divergence of 670 Myr. The 310-Myr date is probably too recent, judging from the fossil evidence, and the 670-Myr date provides a more ancient estimate. We used three values, 310, 400, and 670 Myr,

as calibration points for estimation of the divergence times between closely related Eurotiomycete species.

Our goal was to estimate DNA substitution rates at six independent protein-coding loci and the *ITS* of the *rDNA* repeat unit. To obtain substitution rates, we required estimates for divergence times and genetic distances between sister species. Divergence times between sister species of Eurotiomycete fungi were estimated using NPRS and LF algorithms based on three calibration times corresponding to the three estimates of ES divergence; hence, a total of six rate estimates were calculated for each of the species pairs (table 1). For class 1 chitin synthase (*CHS1*), class 2 chitin synthase (*CHS2*), zinc finger protein (*creA*), and orotidine-5'-phosphate decarboxylase (*pyrG*), four different measures of genetic distance were calculated: DNA substitutions in synonymous sites (K_S), nonsynonymous sites (K_A), the third bases of codons (K_{3rd}), and exons. Introns were not included in these data sets. The modified Nei-Gojobori method implemented in MEGA2.0 (Kumar et al. 2000) was used to estimate K_S and K_A . The Kimura two-parameter model was used for all the other distance measures. Large parts of the data sets for ADP-ribosylation factor (*arf*) and alpha tubulin (*tub1*) are intron sequences, so for these loci four measures of genetic distance were calculated: DNA substitutions in exons, introns, K_{3rd} , and K_S . There were no nonsynonymous substitutions in the *arf* or *tub1* loci.

The most data were available for *CHS1*, which was found in 4 out of 10 pairs of sister species. We used these data to evaluate how estimates of nucleotide substitution varied in different regions of the Eurotiomycete

Table 1
Continued

LOCUS AND SPECIES PAIR	ACCESSION NO. AND LOCUS	SITE	L (bp)	DNA DIS-TANCE ^b	RATE ESTIMATES AT THE GIVEN ES CALIBRATION TIME POINTS (10 ⁻⁹ substitutions site per year)								REFERENCE		
					NPRS				LF						
					310 Myr	400 Myr	670 Myr	310 Myr	400 Myr	670 Myr	310 Myr	400 Myr		670 Myr	
<i>pyrG</i>															
Divergence time estimates . . .															
<i>Aspergillus fumigatus</i> ,	Y11303,														
<i>A. niger</i> ^a	X96734	Exons	834	0.24	34.0 Myr	43.9 Myr	73.6 Myr	15.7 Myr	20.2 Myr	33.9 Myr	33.9 Myr				Weidner et al. (1998)
		Third bases	278	0.79	3.51	2.72	1.62	7.62	5.90	3.52	3.52				
		Synonymous	229.7	1.00	11.60	8.99	5.37	25.18	19.52	11.65	11.65				
		Nonsynonymous	601.3	0.07	14.69	11.38	6.80	31.88	24.70	14.75	14.75				
					1.03	0.08	0.48	2.23	1.73	1.03	1.03				
<i>arf</i>															
Divergence time estimates . . .															
<i>H. capsulatum</i> ,	AF072341,	Exons	280	0.06	99.1 Myr	127.8 Myr	214.1 Myr	24.6 Myr	31.8 Myr	53.3 Myr	53.3 Myr				Kasuga et al. (1999)
<i>B. dermatitidis</i>	AY013310	Introns	174	0.48	0.28	0.22	0.13	1.13	0.88	0.52	0.52				
		Third bases	94	0.15	2.42	1.88	1.12	9.74	7.55	4.51	4.51				
		Synonymous	78.8	0.22	0.78	0.60	0.36	3.12	2.42	1.45	1.45				
					1.11	0.86	0.51	4.46	3.46	2.07	2.07				
<i>tbul</i>															
Divergence time estimates . . .															
<i>H. capsulatum</i> ,	AY013312,	Exons	52	0.10	99.1 Myr	127.8 Myr	214.1 Myr	24.6 Myr	31.8 Myr	53.3 Myr	53.3 Myr				Kasuga et al. (1999)
<i>B. dermatitidis</i>	AY013313	Introns	213	0.49	0.53	0.41	0.24	2.13	1.65	0.98	0.98				
		Third bases	18	0.28	2.49	1.93	1.15	10.01	7.76	4.63	4.63				
		Synonymous	15.6	0.31	1.39	1.08	0.64	5.59	4.33	2.58	2.58				
					1.58	1.23	0.73	6.37	4.94	2.95	2.95				
<i>ITS</i>															
Divergence time estimates . . .															
<i>H. capsulatum</i> ,	AF322377,	ITS1 and ITS2	327	0.21	99.1 Myr	127.8 Myr	214.1 Myr	24.6 Myr	31.8 Myr	53.3 Myr	53.3 Myr				Kasuga et al. (1999)
<i>B. dermatitidis</i>	AF322388	ITS1, ITS2 and 5.8S rDNA	529	0.14	1.08	0.83	0.50	4.33	3.35	2.00	2.00				
					0.73	0.57	0.34	2.84	2.28	1.36	1.36				
					26.8 Myr	34.6 Myr	58.0 Myr	11.5 Myr	14.8 Myr	24.8 Myr	24.8 Myr				
Divergence time estimates . . .															
<i>A. rhoderma incurvatum</i> ,	AF166129,	ITS1 and ITS2	360	0.25	4.58	3.55	2.12	10.71	8.30	4.95	4.95				
<i>T. rubrum</i>	AF170472	ITS1, ITS2 and 5.8S rDNA	518	0.16	3.06	2.37	1.41	7.15	5.54	3.31	3.31				
					106.0 Myr	136.8 Myr	229.1 Myr	45.8 Myr	59.0 Myr	98.9 Myr	98.9 Myr				
Divergence time estimates . . .															
<i>Eremascus albus</i> ,	U18359,	ITS1 and ITS2	337	0.28	1.34	1.04	0.62	3.10	2.40	1.44	1.44				
<i>Ascosphaera apis</i>	U18362	ITS1, ITS2 and 5.8S rDNA	494	0.18	0.87	0.67	0.40	2.01	1.55	0.93	0.93				
					4.5 Myr	5.8 Myr	9.6 Myr	1.7 Myr	2.2 Myr	3.8 Myr	3.8 Myr				
Divergence time estimates . . .															
<i>Penicillium chrysogenum</i> ,	AF034857,	ITS1 and ITS2	342	0.01	1.32	1.02	0.61	3.40	2.63	1.57	1.57				
<i>Eupenicillium crustaceum</i> . .	AF033466	ITS1, ITS2 and 5.8S rDNA	531	0.01	0.85	0.66	0.39	2.18	1.69	1.01	1.01				

Table 1
Continued

LOCUS AND SPECIES PAIR	ACCESSION NO. AND LOCUS	SITE	L (bp)	DNA Dis-TANCE ^b	RATE ESTIMATES AT THE GIVEN ES CALIBRATION TIME POINTS (10 ⁻⁹ substitutions site per year)								REFERENCE				
					NPRS				LF								
					310 Myr	400 Myr	670 Myr	310 Myr	400 Myr	670 Myr	310 Myr	400 Myr		670 Myr			
Divergence time estimates . . .																	
<i>Eurotium rubrum</i> , <i>Aspergillus fumigatus</i>	U18357, AF109330	ITS1 and ITS2 ITS1, ITS2 and 5.8S rDNA	302	0.38	23.8 Myr	30.7 Myr	51.5 Myr	11.5 Myr	15.0 Myr	25.1 Myr	7.50	7.56	4.52	84.0 Myr			
Divergence time estimates . . .																	
<i>Byssoschlamys nivea</i> , <i>Thermoascus crustaceus</i> . . .	U18361, U18353	ITS1 and ITS2 ITS1, ITS2 and 5.8S rDNA	335	0.16	80.8 Myr	104.2 Myr	174.5 Myr	2.12	1.84	0.98	0.98	0.68					

^a Both *pyrG* and *pyrA* from *A. niger* are deposited in GenBank. *pyrA* from *A. niger* showed significantly higher homology to *pyrG* from other fungi than did *pyrG* from *A. niger*. Therefore, we judged that *pyrA* from *A. niger* was homologous to *pyrG*.

^b DNA distance was estimated using the Kimura two-parameter method.

clade and how estimates were influenced by assumptions of rate constancy or rate variability (LF vs. NPRS), the substitution model (ML vs. NJ), or the ES divergence value (310, 400, or 670 Myr). Using data for all four pairs of species, K_S for *CHS1* varied 4.7-fold between the NPRS and LF algorithms, 1.5-fold between the ML–Tamura Nei and NJ–Kimura two-parameter models, and 2.2-fold over the three ES calibration points. The average synonymous substitution rates K_S estimated with NPRS and the three ES values were $7.8 \pm 3.4 \times 10^{-9}$ (310 Myr), $6.1 \pm 2.6 \times 10^{-9}$ (400 Myr), and $3.0 \pm 1.3 \times 10^{-9}$ (670 Myr) ($n = 4$; \pm values are one standard deviation). The average K_S value estimated with LF and an ES of 400 Myr was $28.5 \pm 28.7 \times 10^{-9}$. Most of the variation could be explained by use of the LF algorithm or the NPRS algorithm and its underlying assumptions of rate constancy or variability. In spite of the several sources of variation, K_S estimates ranged only from 10^{-9} to 10^{-8} .

Only one pair of sister species could be used for substitution rate estimation in the remaining protein-coding loci, *CHS2*, *creA*, *pyrG*, *arf*, and *tub1*. K_S values for these genes are also in the range of 10^{-9} – 10^{-8} . The K_S values for the four species pairs at the *CHS1* and one value for each of the *CHS2*, *creA*, *pyrG*, *arf*, and *tub1* loci gave the average of $6.3 \pm 5.4 \times 10^{-9}$ ($n = 9$) when the ES divergence of 400 Myr and NPRS were used. On average, in *CHS1*, *CHS2*, *creA*, and *pyrG*, synonymous substitutions were found to accumulate about 22 times faster ($K_S/K_A = 22.2 \pm 8.4$, $n = 7$) than nonsynonymous substitutions. This value is higher than the average ratio of *Drosophila* genes ($K_S/K_A = 8.2$) but is not an extreme value (Li 1997). The K_S/K_A ratio is positively influenced by both the intensity of negative selection and the population size.

The *arf* and *tub1* data sets consist of exons and introns, for which rates were estimated separately. At *arf* and *tub1* loci, introns mutate 2.2 and 1.6 times faster than synonymous sites and 8.6 and 4.7 times faster than all sites in exons, respectively.

The *ITS* in the *rDNA* repeat unit have been widely used for phylogenetic studies (e.g., Berbee et al. 1995; Cullings, Szaro, and Bruns 1996) and population studies (e.g., O'Donnell 1992; Kasuga et al. 1993). DNA substitution rates were estimated using six pairs of sister species. Unlike with protein-coding genes such as *CHS1* (Bowen et al. 1992) and *RPB2* (Liu, Whelen, and Hall 1999), multiple alignment of DNA sequences from species belonging to different genera was impossible due to the frequent occurrence of indels. The length at the spacer regions (*ITS1* + *ITS2*) varied greatly between the species used in this study, ranging from 302 to 372 bp. DNA substitution rates at the *ITS* region (*ITS1*, *ITS2* + 5.8S rDNA) vary extensively between lineages, and the average was $1.4 \pm 1.3 \times 10^{-9}$ ($n = 5$) when the NPRS and an ES divergence of 400 Myr were used. Despite the large functional difference between the *ITS* region and the protein-coding genes, DNA substitution rates are found to be comparable. In a wide range of plants, substitution rates at the *ITS* region fall between 1.72×10^{-9} and 7.83×10^{-9} (Richardson et al. 2001). Therefore,

estimates for Eurotiomycetes are comparable to the lower values for plants.

Neutral Mutation Rates Across Kingdoms

Overall, neutral mutation rates in protein-coding genes, which were measured as synonymous substitution rates or approximated as substitution rates at the third bases of codons, ranged from 0.9×10^{-9} to 16.7×10^{-9} substitutions per site per year (NPRS and ES = 400 Myr). These values are in the range of DNA substitution rates in most of the protein-coding genes in plants (Gaut et al. 1996), animals (Li 1997), and bacteria (Ochman, Elwyn, and Moran 1999). For example, synonymous substitution rates in cereals are in the range of 5.1×10^{-9} to 7.1×10^{-9} (Wolfe, Sharp, and Li 1989). In mammals, rates were between 1.6×10^{-9} and 6.4×10^{-9} , and in *Drosophila*, rates were between 3.7×10^{-9} and 30.0×10^{-9} (Li 1997; Moriyama and Powell 1997). In *Escherichia coli* and *Salmonella typhimurium*, substitution rates varied from 0.14×10^{-9} to 5.6×10^{-9} (Ochman and Wilson 1987). Although neutral mutation rates vary across loci, their range is surprisingly constant among the four major clades plants, animals, bacteria, and fungi in spite of the enormous differences in cellular organization, body size, generation time, and ecology of these organisms.

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