A Highly Conserved Nuclear Gene for Low-Level Phylogenetics: *Elongation Factor-1α* Recovers Morphology-based Tree for Heliothine Moths

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Molecular systematists need increased access to nuclear genes. Highly conserved, low copy number protein-encoding nuclear genes have attractive features for phylogenetic inference but have heretofore been applied mostly to very ancient divergences. By virtue of their synonymous substitutions, such genes should contain a wealth of information about lower-level taxonomic relationships as well, with the advantage that amino acid conservatism makes both alignment and primer definition straightforward. We tested this postulate for the *elongation factor-1*α (*EF-1*α) gene in the noctuid moth subfamily Heliothinae, which has probably diversified since the middle Tertiary. We sequenced 1,240 bp in 18 taxa representing heliothine groupings strongly supported by previous morphological and allozyme studies. The single most parsimonious gene tree and the neighbor-joining tree for all nucleotides show almost complete concordance with the morphological tree. Homoplasy and pairwise divergence levels are low, transition/transversion ratios are high, and phylogenetic information is spread evenly across gene regions. The *EF-1*α gene and presumably other highly conserved genes hold much promise for phylogenetics of Tertiary age eukaryote groups.

Introduction

The wealth of phylogenetic information present in the nuclear genome remains largely unsampled, despite widespread agreement that secure molecular phylogenetic inference requires multiple, unlinked gene sequences (Miyamoto and Cracraft 1991). Our laboratory has been methodically searching for protein-encoding nuclear gene sequences that will be informative about phylogenetic relationships at various taxonomic levels within animals. Initial screening (Friedlander et al. 1992, 1994) identified *elongation factor-1*α (*EF-1*α), a key protein in the translational elongation process, as a highly promising candidate. The *EF-1*α gene includes >1,300 bp of coding sequence; hence it contains many potential characters. It is free of internal repeats and other obvious features that may complicate analysis.

The *EF-1*α gene is highly conserved in amino acid sequence. For example, the honey bee *Apis mellifera* and the crustacean *Artemia salina,* arthropods whose divergence has been estimated at 550 Myr ago (MYA) (Conway Morris 1993), are 89% identical in amino acid sequence and 73% in nucleotide sequence (Lenstra et al. 1986; Walldorf and Hovemann 1990). Previous systematic applications of this and other highly conserved proteins have concentrated on ancient divergences, such as those among eubacteria, archaebacteria, and eukaryotes or among metazoan phyla (Creti et al. 1991; Rivera and Lake 1992; Hasegawa et al. 1993; Kojima et al. 1993).

It is less widely appreciated that highly conserved, protein-encoding, nuclear genes may also be phylogenetically informative at much lower taxonomic levels, by virtue of their more rapidly evolving synonymous substitutions (but see Slade et al. 1994). Indeed, the relative ease and confidence of multiple sequence alignments and of PCR primer definition afforded by the high conservation of codons may give such genes a significant advantage over more rapidly evolving sequences, including introns, for which indels and substitutions can complicate these essential tasks. We have tested the phylogenetic utility of *EF-1*α sequences within a recently evolved group, the noctuid moth subfamily Heliothinae (Insecta: Lepidoptera: Noctuidae), using the criterion of "concordance" (Friedlander et al. 1992, 1994), that is, the ability of *EF-1*α characters to recover groupings strongly supported by previous evidence (Miyamoto and Cracraft 1991).

The Heliothinae are a cosmopolitan group of about 400 species (Matthews 1991; Mitter et al. 1993). The larvae attack the flowers and fruits of herbaceous plants

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and include a number of the world's most injurious crop pests, such as the corn earworm (*Helicoverpa zea*) and its Old World relatives and the tobacco budworm (*Heliothis virescens*) and related New World species.

Previous morphological and allozyme studies of heliothine systematics leave many problems unsolved but provide several well-supported groupings by which the potential informativeness of nucleotide character sets can be judged. As depicted in figure 1*C*, there is evidence for monophyly of the subfamily, of a number of generic and subgeneric taxa, and of a "*Heliothis* group" comprising about half the species and all the major pests. Recovery of a similar arrangement by *EF-1α* sequences would be strong evidence that this gene carries information on heliothine phylogeny.

Material and Methods

To test for such concordance, we sequenced 1,240 bp of the *EF-1α* coding region from 15 species and subspecies within Heliothinae, including multiple representatives of all major, recognized groups (fig. 1*B*). In addition, three species (*Spodoptera frugiperda, Basilodes chrysopis, Trichoplusia ni*) from related subfamilies served as outgroups. The 18 species sequenced and geographic sources of the material for each are listed in table 1. Field-collected adults were killed and transported to the laboratory either in liquid nitrogen or in 100% ethanol kept at 0° or −20°C, then stored at −80°C until used. The wings and, in most cases, the genitalia were saved as vouchers at the Department of Entomology, University of Maryland at College Park. One or two specimens were used for each species.

Five primer pairs were developed (table 2), starting from comparisons of published arthropod and vertebrate sequences. Little modification was required for heliothines, and each primer pair yielded a single, major, intron-less PCR product. Our observations do not preclude the presence of introns within untranslated regions or within the small portion of the coding region not amplified.

DNA was extracted and purified either following Sambrook et al. (1989) with slight modifications or using a commercially available DNA/RNA isolation kit (U.S. Biochemical). Symmetric PCR was performed using five pairs of primers that amplify most (89.3% of total in *Drosophila*) of the *EF-1α* gene. Thermocycle conditions were 95°C for 30 s (3 min for first cycle only), 55°C for 30 s, and 72°C for 2 min (10 min for last cycle only) for a total of 30 cycles, using a Perkin-Elmer GeneAmp PCR System 9600 and MicroAmp tubes. Initial studies used a second, asymmetric PCR amplification to obtain single-stranded templates for manual dideoxy sequencing. Subsequently, we switched to direct sequencing of symmetric PCR products using an Applied Biosystems 373 DNA sequencer. Both DNA strands were sequenced.

Sequences were assembled using the software program STADEN (Staden 1992). Alignments required no gaps and were determined by inspection using the Genetic Data Environment software package (GDE 2.2; Smith et al. 1994) from the Harvard Genome Laboratory. Parsimony analysis and calculation of pairwise divergences for both nucleotide and amino acid sequences were performed in PAUP 3.1.1 (Swofford 1993). Each parsimony analysis employed a heuristic search, using TBR branch swappings, with "simple," "closest," and "random (100 replications)" taxon addition. The initial unweighted parsimony analysis of the full nucleotide data set also included 500 bootstrap replications (Felsenstein 1985) and calculation of the decay index (Bremer 1988; Donoghue et al. 1992) for each group. Subsequent variations on this analysis included additions and deletions of one or more outgroups and character weightings based on (*a*) codon position (nt1:nt2:nt3 = 10:10:1), (*b*) base change type (transversions:transitions = 2:1), (*c*) retention index on the initial tree, using successive weighting (Farris, 1969; the reweight characters option in PAUP), and (*d*) number of character states (2-state:3-state:4-state = 3:2:1). Separate analyses were also carried out on nt3 and on characters undergoing only synonymous changes.

For comparison, a neighbor-joining tree (Saitou and Nei 1987) was calculated based on the Kimura two-parameter distance (Kimura 1980), using PHYLIP 3.4 (Felsenstein 1992).

Results and Discussion

Unweighted analysis of all nucleotides yields a single most parsimonious tree (fig. 1*B*). The same result was obtained under all character weightings. An identical tree for Heliothinae also results when representatives of a different family (*Lymantria dispar*, Noctuoidea: Lymantriidae) and superfamily (*Manduca sexta*, Bombycoidea: Sphingidae) are added to the three noctuid outgroups or when all nonheliothines are deleted and the *Adisura* species is the outgroup. The neighbor-joining method also produces the same tree, except within the *Australothis*/*Helicoverpa* lineage, where the branching order is (*Helicoverpa zea*(*H. armigera conferta*(*H. armigera armigera, Australothis rubrescens*))).

Of the 1,240 nucleotide sites, 182 (14.7%) are variable and 104 (8.4%) are informative for parsimony analysis. Most, but not all, of the phylogenetic information lies in the third codon position, and most substitutions (281/299) are synonymous. First, second, and third positions contribute 17, 6, and 276 substitutions, respectively, to the total. Analysis of nt3 or synonymous sites

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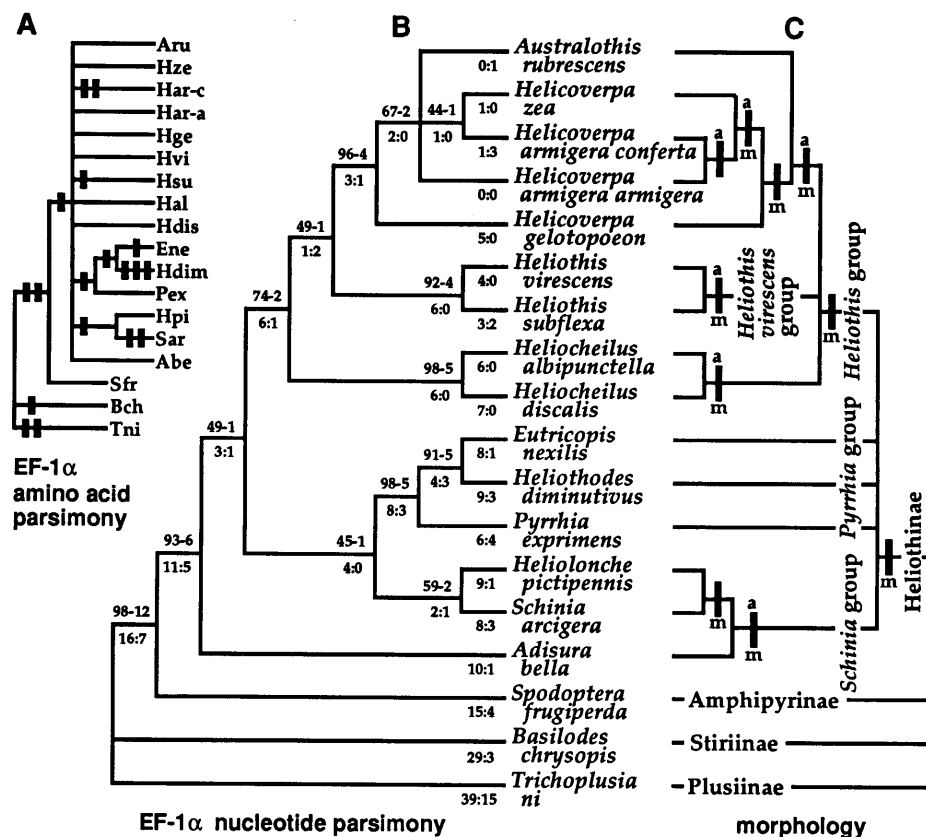

FIG. 1.—Comparison of heliothine relationships based on parsimony analysis of *EF-1α* and morphology. *A*, The single most parsimonious tree based on amino acid changes (18 total) in *EF-1α* (consistency index = 0.944, retention index = 0.875). Within Heliothinae alone, there are no homoplasious amino acid changes. Tick marks identify amino acid changes. Terminal taxa are identified by a three- or four-letter code in which the first letter corresponds to the first letter of the genus name and the others to the first letters of the species and subspecies names. *B*, The single most parsimonious tree found for all nucleotides (unweighted). Bootstrap values (500 replicates) followed by decay indices are placed above internal branches. Numbers of transitions followed by transversions under Acctran optimization are placed below branches. Gene tree length = 299; consistency index = 0.666; retention index = 0.608. *C*, Conservative summary of relationships in Heliothinae as currently understood, following the morphological cladistic study of Matthews (1991; Mitter et al. 1993), which extended earlier work by Hardwick (1965, 1970). Monophyly of groupings marked *m* is supported by clear morphological synapomorphies. Groups marked *a* are supported by allozyme analysis (C. Mitter and R. W. Poole, unpublished data). Poole et al. (1993) report additional synapomorphies for the *Heliothis virescens* group. Relationships within *Helicoverpa*, consistent with Hardwick (1965), are from unpublished morphological and allozyme studies (C. Mitter and R. W. Poole).

alone yields eight most parsimonious trees, including the one in figure 1*B*.

Amino acid changes alone (fig. 1*A*) support monophyly of Heliothinae, *Heliolonche* + *Schinia*, the *Pyrrhia* group, and *Eutricopis* + *Heliothodes*. These groupings are completely concordant with the nucleotide tree, and no homoplasy is required within Heliothinae. However, much of the tree is left unresolved.

Synonymous divergences in Heliothinae appear to be well below the saturation level. Total nucleotide, pairwise differences (fig. 2) range from 0.1% to 1.0% within a genus and 1.4% to 4.0% between heliothine genera. The ratio of transition to transversion changes over the entire, most parsimonious tree is 4.25:1 (242:57). A plot by nucleotide site of the number of changes required on the most parsimonious tree (fig. 3) reveals that 58% (105/182) of variable, third-codon positions show only one change, while 28% (51/182) show only two changes. The distribution of changes appears uniform across the gene except for a slightly lower frequency in the first 200 nucleotides, suggesting that phylogenetic information is spread through the entire sequence.

Interspecific variation in base composition is low for total nucleotides, as well as for nt3 alone. For example, within Heliothinae for all nucleotides, no taxon varies from any other by more than 6.1%, 5.8%, 2.0%, and 6.6% of the mean for A, C, G, and T nucleotides, respectively; means are 24.5%, 29.2%, 25.0%, and 21.2%, respectively.

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Table 1
Geographic Sources for Specimens Analyzed

Species	Source[a]
Australothis rubrescens	Australia, Toowoomba (36) [U20124]
Helicoverpa zea[b]	U.S.A., Mississippi, Stoneville [U20136]
H. armigera conferta	Australia (124) [U20128]
H. armigera armigera[b]	Thailand [U20129]
H. gelotopoeon	Argentina, lab colony (123) [U20132]
Heliothis virescens[b]	U.S.A., Mississippi, Stoneville, USDA lab colony [U20135]
H. subflexa	U.S.A., Florida, lab colony (122) [U20134]
Heliocheilus albipunctella	Mali, Mourdiah (121) [U20127]
H. discalis	Mali, Mourdiah (120) [U20131]
Eutricopis nexilis	U.S.A., California, Sonora Pass (116) [U20126]
Heliothodes diminutivus	U.S.A., California, Cobb Mtn. (117) [U20130]
Pyrrhia exprimens	U.S.A., Maryland (38) [U20137]
Heliolonche pictipennis	U.S.A., California, Kelso (30) [U20133]
Schinia arcigera	U.S.A., Texas, Houston (118) [U20138]
Adisura bella	Mali, Mourdiah (119) [U20123]
Spodoptera frugiperda[b,c]	U.S.A., Maryland, College Park, lab colony [U20139]
Basilodes chrysopis	U.S.A., Texas, El Paso (126) [U20125]
Trichoplusia ni[b,c]	U.S.A., Maryland, College Park, lab colony [U20140]

NOTE—With exceptions noted, we used wild-caught animals.
[a] Voucher numbers are in parentheses and GenBank accession numbers for their *EF-1α* sequences are in brackets.
[b] Two specimens were pooled for analysis.
[c] Pupae were used instead of adults.

The *EF-1α* gene tree is almost completely concordant with that based on morphology, corroborating both the phylogenetic informativeness of *EF-1α* and the main previous conclusions about heliothine relationships. Strong support (high bootstrap and decay index values) is evident for the monophyly of the subfamily and for a number of its subgroups, such as the *Heliothis* group, *Heliocheilus,* the *Heliothis virescens* group and *Helicoverpa* + *Australothis.* Departures of the *EF-1α* tree from the morphology tree are few, minor, and not strongly supported. Removal of *Australothis* from within *Helicoverpa* requires only two additional steps; *EF-1α* variation is probably insufficient within this clade to resolve relationships. Inclusion of *Adisura* in the *Schinia* group requires only two additional steps; resolution in this group may require better sampling of *Schinia,* by far the largest heliothine genus (~150 species).

The *EF-1α* gene also shows promise for clarifying relationships currently judged problematic. For example, there is very strong support (fig. 2) for monophyly of the

Table 2
Sequences of *EF-1α* Primers Used in this Study

M3 *CAGGAAACAGCTATGACC*CACAT(CT)AACATTGTCGT(CG)AT(CT)GG [2103]
rcM44.9 *TGTAAAACGACGGCCAGT*CTTGATGAAATC(CT)CTGTGTCC [2342]
M44-1 *CAGGAAACAGCTATGACC*GCTGAGCG(CT)GA(GA)CGTGGTATCAC [2277]
rcM51-1 *TGTAAAACGACGGCCAGT*CAT(GA)TTGTC(GT)CCGTGCCA(GT)CC [2645]
M46-1 *CAGGAAACAGCTATGACC*GAGGAAAT(CT)AA(GA)AAGGAAG [2582]
rcM52.6 *TGTAAAACGACGGCCAGT*GC(CT)TCGTGGTGCAT(CT)TC(GC)AC [2936]
M51.9 *CAGGAAACAGCTATGACC*CA(GA)GACGTATACAAAATCGG [2832]
rcM53-2 *TGTAAAACGACGGCCAGT*GCAATGTG(GA)GCIGTGTGGCA [3149]
M52.7 *CAGGAAACAGCTATGACC*GTCAAGGA(GA)(CT)TGCGTCGTGG [3030]
rcM4 *TGTAAAACGACGGCCAGT*ACAGC(CGA)AC(GT)GT(TC)TG(CT)CTCAT(AG)TC [3344]

NOTE.—Degenerate positions are identified by placing the appropriate nucleotides within parentheses. The 5′-most 18 nucleotides (in italics) of each primer correspond to the "M13 reverse" or "M13 (−21) forward" primer sites of M13 bacteriophage, added to facilitate automated sequencing; *rc* reverse complement, which indicates that the primer binds to the sense strand of the DNA. Numbers in brackets at the 3′ end of each primer refer to nucleotide position relative to the *Drosophila melanogaster* sequence.

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	Aru	Hze	Har-c	Har-a	Hge	Hvi	Hsu	Hal	Hdis	Ene	Hdim	Pex	Hpi	Sar	Abe	Sfr	Bch	Tni
Aru		0.2	0.5	0.1	0.6	1.5	1.5	1.8	1.9	3.1	3.6	2.9	2.2	2.0	2.3	3.5	6.4	6.5
Hze			0.4	0.2	0.8	1.8	1.9	1.9	2.0	3.5	4.0	3.2	2.4	2.2	2.7	3.9	6.7	6.5
Har-c				0.4	0.6	1.5	1.5	1.6	1.7	3.0	3.5	2.7	2.0	1.9	2.2	3.4	6.3	6.3
Har-a					0.6	1.4	1.5	1.7	1.8	3.1	3.5	2.8	2.1	1.9	2.3	3.5	6.4	6.4
Hge						1.6	1.7	1.9	2.0	3.3	3.8	3.0	2.2	2.3	2.4	3.7	6.8	6.5
Hvi							0.7	1.9	1.9	3.2	3.7	2.9	1.9	2.2	2.4	3.1	6.4	6.5
Hsu								2.2	2.2	3.4	3.9	3.1	2.2	2.4	2.5	3.3	6.5	6.7
Hal									1.0	3.1	3.7	3.1	2.4	2.3	2.6	4.3	6.9	6.5
Hdis										3.1	3.7	2.8	2.7	2.4	2.6	3.8	6.5	6.1
Ene											1.7	2.1	2.9	2.8	3.1	4.7	5.9	7.4
Hdim												2.3	3.2	3.0	3.5	4.6	6.3	8.0
Pex													2.7	2.7	2.8	4.2	6.3	7.3
Hpi														1.7	2.4	3.8	6.3	6.9
Sar															2.7	3.7	6.2	6.7
Abe																3.7	6.2	6.2
Sfr																	5.9	6.5
Bch																		6.9
Tni																		

$\bar{x}$=0.5% within *Helicoverpa*

$\bar{x}$=0.7% within *Heliothis virescens* group

$\bar{x}$=1.0% within *Heliocheilus*

$\bar{x}$=2.0% between *Pyrrhia* group genera

$\bar{x}$=2.3% between *Schinia* group genera

FIG. 2.—Percent divergence for pairwise comparisons of 18 *EF-1α* sequences (all nucleotides). Taxon abbreviations as in fig. 1*A*.

Pyrrhia group as sampled here, supporting an earlier postulate by Hardwick (1965, 1970). The *EF-1α* tree also suggests a close relationship between the corn earworm complex (*Helicoverpa*) and the tobacco budworm (*Heliothis virescens*) group, in agreement with at least one morphological character (Poole et al. 1993), implying that the life-history traits characteristic of both these pests may represent shared inheritance. Currently, we are sequencing additional taxa, in hopes of resolving this and other unsettled questions in heliothine phylogeny. Initial results of a broader survey suggest that *EF-1α* will also be informative about relationships of Heliothinae to other noctuid subfamilies.

For predicting the applicability of *EF-1α* synonymous changes to other groups, it is desirable to estimate the age of Heliothinae. Fossils are lacking but an indication of age can be extracted from a survey of 26 allozyme loci in 30 heliothine species (unpublished data), including all those analyzed for *EF-1α* except the *Pyrrhia* group. Average values of Nei's genetic distance were ~0.7 between *Helicoverpa* and the *Heliothis virescens* group, ~0.9 between these and *Heliocheilus,* and ~1.1 between the *Schinia* group and the *Heliothis* clade. The extreme values from extensive compilations of suggested allozyme clock calibrations (Avise and Aquadro 1982; Thorpe 1982) would place basal heliothine divergence between ~1 and ~20 MYA (but note that the date based on calibrations for Carnivora by Wayne et al. [1991] would be 50 MYA or older). It is thus a reasonable inference that heliothines have diversified in the Cenozoic, probably in the second half of this era. The low divergence levels within Heliothinae suggest that *EF1-α* will also resolve somewhat older divergences.

The absence of introns within the coding regions sequenced and the apparent single-copy nature of *EF-1α* have made this gene particularly attractive for systematics of Heliothinae. The *EF-1α* gene is likely to be useful within many other metazoan groups. Like Heliothinae, and apparently Lepidoptera generally (T. P. Friedlander, unpublished data), the honey bee also has a single *EF-1α* gene, although its coding region is interrupted by two relatively small introns (Walldorf and Hovemann 1990). The fly *Drosophila melanogaster* has two copies of *EF-1α,* called F1 and F2, that are approximately 10% divergent in amino acid sequence (Hovemann et al. 1988). The F1 copy has no introns interrupting its coding region, although the 5′ untranslated region has one, whereas the F2 copy has two introns each in its 5′ untranslated and coding regions. In this case, the presence of introns should be advantageous for systematics by permitting convenient separation of gene-specific PCR amplification products. Brine shrimp has been reported to have up to four copies, although no more than a single copy, whose coding region is interrupted by four introns, has been clearly demonstrated (Lenstra et al. 1986). Outside Arthropoda, total *EF-1α* RNA from a mollusc, two annelids, and a vestimentiferan has been partially sequenced via their cDNAs, a result consistent with a single type of sequence (Kojima et al. 1993). The frog *Xenopus laevis* appears to have at least three *EF-1α* genes with stage-specific patterns of expression and distinct intronic structures, plus multiple pseudogenes (Djé et al. 1990; Abdallah et al. 1991). This complexity may be related to the apparently polyploid germline genome of *Xenopus laevis* (Cannatella and de Sá 1993). Chicken (Wang et al. 1994), and possibly human beings (Brands et al. 1986) and rabbit (Cavallius and Merrick 1992), appear to have single gene copies. These findings, coupled with the phylogenetic informativeness of *EF-1α* demonstrated in this study and in others at higher taxonomic levels (Creti et al. 1991; Riv-

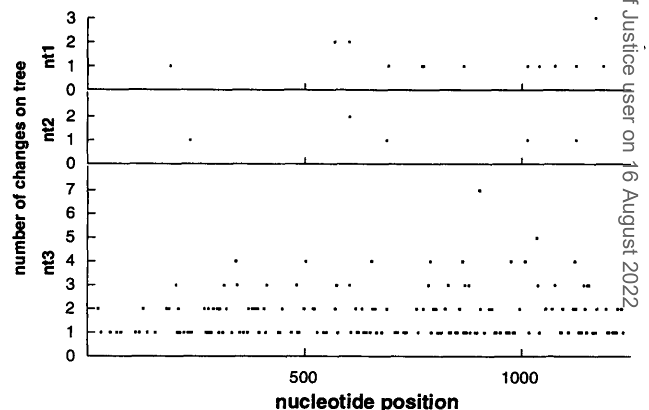

FIG. 3.—Spatial distribution of variable nucleotide sites separated according to codon position (nt1, top graph; nt2, middle graph; nt3, bottom graph). For each position (5′ to 3′), the minimum number of changes on the tree in fig. 1*B* is plotted.

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era and Lake 1992; Hasegawa et al. 1993; Kojima et al. 1993), suggest that this gene will, with due caution, prove widely applicable to systematics.

Conclusions

This study confirms that synonymous substitutions in a highly conserved, protein encoding nuclear gene can provide characters of high quality and effective quantity for phylogenetic inference at lower taxonomic levels. Primer design from distantly related published sequences was straightforward, eliminating a difficulty that has heretofore limited most low-level sequence-based phylogenetic studies to organellar genes. The *EF-1α* gene and presumably other highly conserved, protein encoding nuclear genes should prove widely applicable to the phylogenetics of eukaryotic groups that diverged in the middle to late Tertiary.

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