Phylogenetic Utility and Evidence for Multiple Copies of Elongation Factor-1α in the Spider Genus Habronattus (Araneae: Salticidae)

Marshal C. Hedin[1] *and Wayne P. Maddison*

Department of Ecology and Evolutionary Biology, University of Arizona

In the continuing quest for informative genes for use in molecular systematics, the protein-coding gene Elongation factor–1α (EF-1α) has rapidly become one of the most prevalent ''single-copy'' nuclear genes utilized, particularly in arthropods. This paper explores the molecular evolutionary dynamics and phylogenetic utility of EF-1α in the salticid spider genus Habronattus. As has been reported for other arthropod lineages, our studies indicate that multiple (two) copies of EF-1α exist in Habronattus. These copies differ in intron structure and thus in size, making it possible to easily separate PCR amplification products. We present data for an intronless EF-1α copy for three Habronattus species. The presence of nonsense mutations and generally elevated rates of amino acid change suggest that this copy is evolving under relaxed functional constraints in Habronattus. A larger taxon sample (50 species plus outgroups) is presented for an EF-1α copy that includes both intron and exon regions. Characteristics of both regions suggest that this is a functional, orthologous copy in the species sampled. Maximum-likelihood relative-rate comparisons show that exon third codon sites are evolving more than 100 times as fast as second codon sites in these sequences and that intron sites are evolving about twice as fast as exon third sites. In combination, the EF-1α data provide robust, species-level phylogenetic signal that is largely congruent with morphologically well supported areas of Habronattus phylogeny. The recovery of some novel clades, and the unexpected fragmentation of others, suggests areas requiring further phylogenetic attention.

Introduction

Recent progress in spider systematics has been most influenced by two advances: the development of new morphological character sets (made accessible through SEM) and the growth of cladistic thought (see Coddington and Levi 1991; Griswold et al. 1999). The development and general impact of molecular systematics has lagged somewhat behind, although this appears to be changing. This is particularly true at lower taxonomic levels, with many recent phylogeographic and species-level phylogenetic studies using molecular data (e.g., Gillespie, Croom, and Hasty 1997; Zehethofer and Sturmbauer 1998; Masta 2000; Bond et al. 2001; Hedin 2001). To date, essentially all studies have relied on a single gene region (mitochondrial) and thus are potentially subject to the various pitfalls that such analyses present (see Maddison [1997] and others). More, different types of molecular markers need to be developed for the same reasons that we should be interested in using both molecular and morphological evidence in a complementary manner. New character sets represent more evidence, independent evidence, and potentially heterogeneous evidence (i.e., with different rate dynamics, thus resolving different depths of phylogenetic divergence).

The protein-coding gene Elongation factor–1α (EF-1α) is rapidly becoming one of the most prevalent ''single-copy'' nuclear genes utilized in arthropod molecular systematics. EF-1α is a key and conservative element in protein synthesis, thus facilitating universal use and phylogenetic data collection. To date, studies using EF-1α data have suggested a broad, but perhaps bimodal, distribution of phylogenetic utility. Utility has been impressive for Tertiary age divergences, with most information coming from silent substitutions at third codon positions (e.g., Cho et al. 1995; Reed and Sperling 1999). Using a combination of intron and exon sequence data, Danforth, Sauquet, and Packer (1999) extend this utility to the species level in halictine bees. Third-position saturation, coupled with amino acid conservation, appears to limit utility at ''intermediate'' levels (e.g., Mesozoic divergences), but amino acids are again informative at deep levels (e.g., among arthropods; see Regier and Shultz 1997; Shultz and Regier 2000). Based on the generally encouraging results from the handful of published studies, Caterino, Cho, and Sperling (2000) suggest that EF-1α should be a ''focal gene'' in insect molecular systematics, contingent on the prevalence and potential difficulties associated with multiple gene copies.

Here we report on the molecular evolutionary dynamics and phylogenetic utility of EF-1α in the spider genus Habronattus. Habronattus is one of the most species-rich spider genera in the Americas, including over 90 described species distributed from Canada south to northern South America (Griswold 1987). The genus is remarkable in other respects, with interesting patterns of sex chromosomal evolution (Maddison 1982), fine-scale morphological and behavioral divergence (Maddison and McMahon 2000), and a diversity of communication modalities (e.g., visual, stridulatory, etc.; Peckham and Peckham 1889; Maddison and Stratton 1988). Morphology-based phylogenetic analyses have been conducted by Griswold (1987) and are summarized in figure 1.

Two apparent phylogenetic patterns are important to the focus of this paper. The first pattern is the ''bundling'' of species into species groups. The character support for some of these groups is so strong (e.g., the *americanus,*
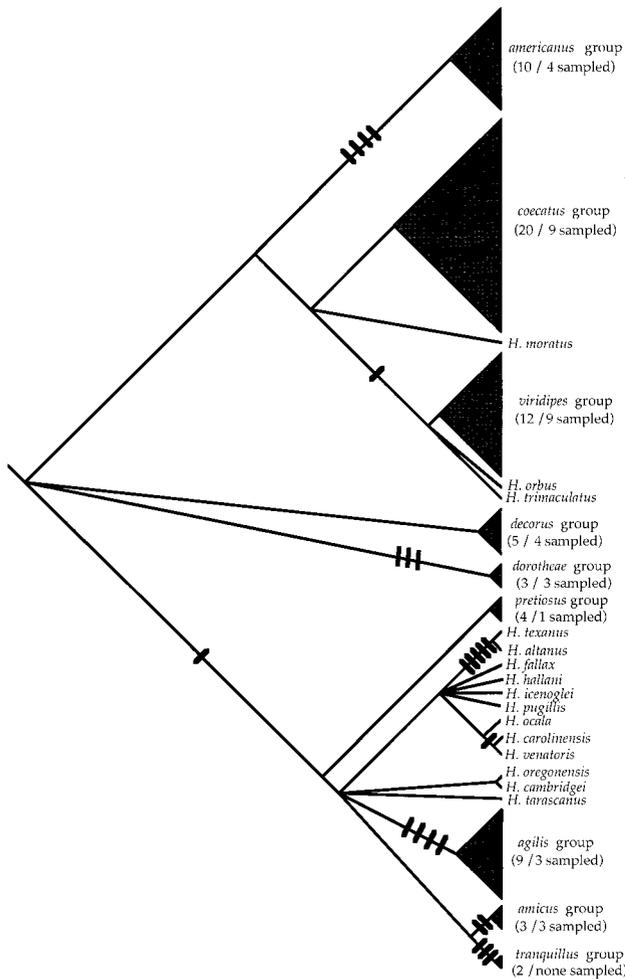
FIG. 1.—Morphological phylogeny of Habronattus, with corresponding DNA samples. The phylogeny shown, based on a mental analysis of 57 "intuitively selected characters," corresponds to figure 10 of Griswold (1987). The nine species groups recognized by Griswold are shaded. In parentheses are the number of recognized species/ the number of species sampled. Species identified by name were not placed into species groups by Griswold. Bars on branches represent hypothesized nonhomoplastic synapomorphies. Resolved groupings without bars are supported by characters that are either variable in the terminal taxa, involving parallel gains or losses, or of uncertain homology (see Griswold 1987).

dorotheae, and *agilis* groups) that we would be surprised if molecular analyses failed to recover coincident monophyletic lineages. Alternatively, there are species groups lacking robust character support (e.g., the *decorus* and *pretiosus* groups), where independent analyses are needed to confirm or perhaps refute hypothesized groupings. Furthermore, relationships among groups, particularly near the base of the genus, are currently poorly understood (fig. 1). A second interesting pattern, essentially opposite that of the "bundling" pattern, is the presence of distinctive Habronattus species lacking clear phylogenetic relatives (e.g., *H. tarascanus, H. pugillis, H. hallani*; fig. 1). It would be interesting to know if such species are components of "cryptic" species groups (i.e., strongly supported molecular clades that lack obvious morphological synapomorphies), which would thus comprise sets of very distinctive taxa. Of course, it might be the case that such phylogenetic

"stragglers" are real, which would have equally interesting implications for speciation/extinction dynamics in Habronattus.

Materials and Methods

We gathered sequences from 50 Habronattus species, representing a large percentage of the phylogenetic stragglers, and multiple species for all but two of Griswold's (1987) nine recognized species groups (fig. 1 and table 1). Within species groups we targeted taxa hypothesized to be phylogenetically distant (i.e., species from opposite sides of a proposed root node). Although within-species geographic variation is prevalent in Habronattus, we sampled only a single population per species for this study. Sequences from two species of Pellenes and a single species of Hawaiian "Sandalodes" were included as outgroups, following molecular phylogenetic analyses of salticid relationships that strongly support a ((Pellenes, Habronattus) "Sandalodes") grouping (unpublished data).

Genomic DNA was extracted from either fresh or ETOH-preserved leg tissue using the CTAB protocol of Shahjahan et al. (1995). Otherwise-intact spiders are preserved as alcohol voucher specimens at −80°C in the personal collection of W.P.M. Initial EF-1α sequences were generated using universal degenerate primers (Cho et al. 1995) available in the UBC Insect Nuclear DNA Oligonucleotide Set. From these preliminary sequences, spider-devoted primers were designed by identifying conserved regions shared across Habronattus and available spider EF-1α exon sequences (Regier and Shultz 1997). For most genomic templates, these primers (M46-1SPID [5′-GAG GAA ATC AAG AAG GAA G-3′] and rc1007-1 [5′-GAC CTG AGC AAA GAA TTC TTG AGT AC-3′]) amplify a single fragment approximately 700 bp in length. A small percentage of templates produced multiple bands; these products were size-separated for further analysis. PCR cycle parameters were as follows: 2 min 30 s initial 95°C denaturation, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C, and 45 s at 72°C + 3 s per cycle, followed by a final 72°C extension for 10 min. All PCR reactions included TaqStart Antibody (Clontech Laboratories) at dilutions suggested by the manufacturer.

Double-stranded PCR products were polyacrylamide gel-purified (Sambrook, Fritsch, and Maniatis 1989) and directly sequenced using ABI dye chemistry on an ABI 377 machine. Both strands were determined for most templates using PCR primers as sequencing primers. T-rich regions at the 3′ end of intron I (see below and fig. 2) caused premature stops in some sequencing reads; sequences for these templates were thus determined using single reads from opposite sides of the intron. Because we were uncertain of the exact number of repeated T's in some of these sequences, we excluded a 6-bp region at the 3′ end of intron I from all phylogenetic analyses. Sites including two peaks of equivalent intensity were interpreted as heterozygosity and entered into the phylogenetic matrix using IUBMB ambiguity codes. Of approximately 38,000 total aligned sites, 53

Table 1
Taxon Sample

Species	Species Group[a]	Locality[b]	Accession No.
Sandalodes	—	USA: Hawaii, Volcano	AF359058
Pellenes longimanus	—	USA: Texas, Benzten-Rio Grande SP	AF359059
Pellenes shoshonensis	—	USA: California, White Mountains	AF359060
Habronattus alachua	AGIL	USA: Florida, Ordway	AF359061
Habronattus cognatus	AGIL	USA: Texas, vic. Giraween	AF359062
Habronattus conjunctus	AGIL	USA: Arizona, Whetstone Mountains	AF359063
Habronattus amicus	AMIC	USA: California, near Old Station	AF359064
Habronattus ustulatus	AMIC	USA: Arizona, Maricopa Wilderness	AF359065
Habronattus signatus	AMIC	USA: California, near Ocotillo	AF359066
Habronattus geronimoi	DORO	USA: Arizona, Huachuca Mountains	AF359067
Habronattus dorotheae	DORO	Mexico: Jalisco, near Laguna Sayula	AF359068
Habronattus huastecanus	DORO	Mexico: Chiapas, Huamuche	AF359069
Habronattus cockerelli	DECO	USA: New Mexico, Sacramento Mountains	AF359070
Habronattus cf. *decorus*	DECO	USA: Florida, Alachua County, State Route 325	AF359071
Habronattus banksi	DECO	Costa Rica: Guanacaste, Palo Verde NP	AF359072
Habronattus cf. *sugillatus*	DECO	Mexico: Nayarit, west of Tepic	AF359073
Habronattus cf. *pochtecanus*	PRET	Mexico: Jalisco, Los Yesos	AF359074
Habronattus carolinensis	MISC[c]	USA: Florida, vic. Florahome	AF359075
Habronattus venatoris	MISC	USA: New Mexico, upper Pecos River	AF359076
Habronattus ocala	MISC	USA: Florida, Ocala NF	AF359077
Habronattus altanus	MISC	USA: Arizona, San Fransisco Mountains	AF359078
Habronattus texanus	MISC	USA: Texas, Benzten-Rio Grande SP	AF359079
Habronattus fallax	MISC	USA: Texas, vic. Giraween	AF359080
Habronattus hallani	MISC	USA: Arizona, Tucson	AF359081
Habronattus oregonensis	MISC	USA: Arizona, Santa Rita Mountains	AF359082
Habronattus pugillis	MISC	USA: Arizona, Patagonia Mountains	AF359083
Habronattus cambridgei	MISC	Mexico: Guerrero, near Ajuchitlán	AF359084
Habronattus icenoglei	MISC	USA: Arizona, vic. Mohawk Mountains	AF359085
Habronattus tarascanus	MISC	Mexico: Michoacán, near Tzararacua	AF359086
Habronattus americanus	AMER	USA: California, White Mountains	AF359087
Habronattus sansoni	AMER	Canada: British Columbia, Bull River	AF359088
Habronattus kawini	AMER	Mexico: Baja California Norte, Laguna Hanson	AF359089
Habronattus tarsalis	AMER	USA: California, upper Klamath River	AF359090
Habronattus zebraneus	COEC	Mexico: Morelos, near Xochicalco	AF359091
Habronattus ammophilus	COEC	Mexico: Baja California Sur, vic. Pichilingue	AF359092
Habronattus cf. *anepsius*	COEC	Mexico: Baja California Sur, Isla Magdalena	AF359093
Habronattus ballatoris	COEC	USA: California, near Orleans	AF359094
Habronattus borealis	COEC	Canada: Ontario, Hamilton	AF359095
Habronattus coecatus	COEC	USA: Georgia, Oconee River	AF359096
Habronattus pyrrithrix	COEC	USA: Arizona, Tucson	AF359097
Habronattus mexicanus	COEC	USA: Texas, lower Pecos River	AF359098
Habronattus virgulatus	COEC	USA: Arizona, lower Santa Catalina Mountains	AF359099
Habronattus calcaratus calcaratus	VIRD	USA: Florida, Ordway	AF359100
Habronattus calcaratus maddisoni	VIRD	USA: Tennessee, Cedars of Lebanon SP	AF359101
Habronattus jucundus	VIRD	USA: Idaho, northeast of Lowell	AF359102
Habronattus viridipes	VIRD	USA: Texas, near Armstrong	AF359103
Habronattus notialis	VIRD	USA: Florida, near Boulogne	AF359104
Habronattus forticulus	VIRD	USA: Texas, near Austin	AF359105
Habronattus aztecanus	VIRD	Mexico: Nayarit, near San Blas	AF359106
Habronattus divaricatus	VIRD	Mexico: Baja California Sur, Sierra La Laguna	AF359107
Habronattus velivolus	VIRD	Mexico: Jalisco, near Tequila	AF359108
Habronattus trimaculatus	VIRD[d]	USA: Florida, southwest of Archer	AF359109
Habronattus orbus	VIRD[d]	USA: Missouri, Hahn SP	AF359110
Habronattus moratus	VIRD[d]	USA: Texas, north of Rio Grande City	AF359111

[a] Species group designations follow Griswold (1987).

[b] More detailed locality information is available on request from the authors.

[c] Taxa labeled as MISC were considered ''species *incertae sedis*'' by Griswold (1987) and were not placed into any formal species group.

[d] Taxa tentatively placed in the *viridipes* species group (Griswold 1987).

sites were scored as ambiguous (31 exon sites, 22 intron sites).

Parsimony constituted the primary phylogenetic estimation procedure, carried out using the program PAUP*, version 4.0b2a–b4a (Swofford 1999), in combination with MacClade, version 4 (Maddison and Maddison 2000). Searches were heuristic with TBR branch

rearrangement, using stepwise taxon addition from 500 random replicates, saving no more than 500 most-parsimonious (MP) trees per replicate. Relative clade support was assessed using nonparametric bootstrap analyses (Felsenstein 1985). Bootstrap analyses comprised 200 full heuristic search replicates, each including five random-addition sequence searches with TBR branch
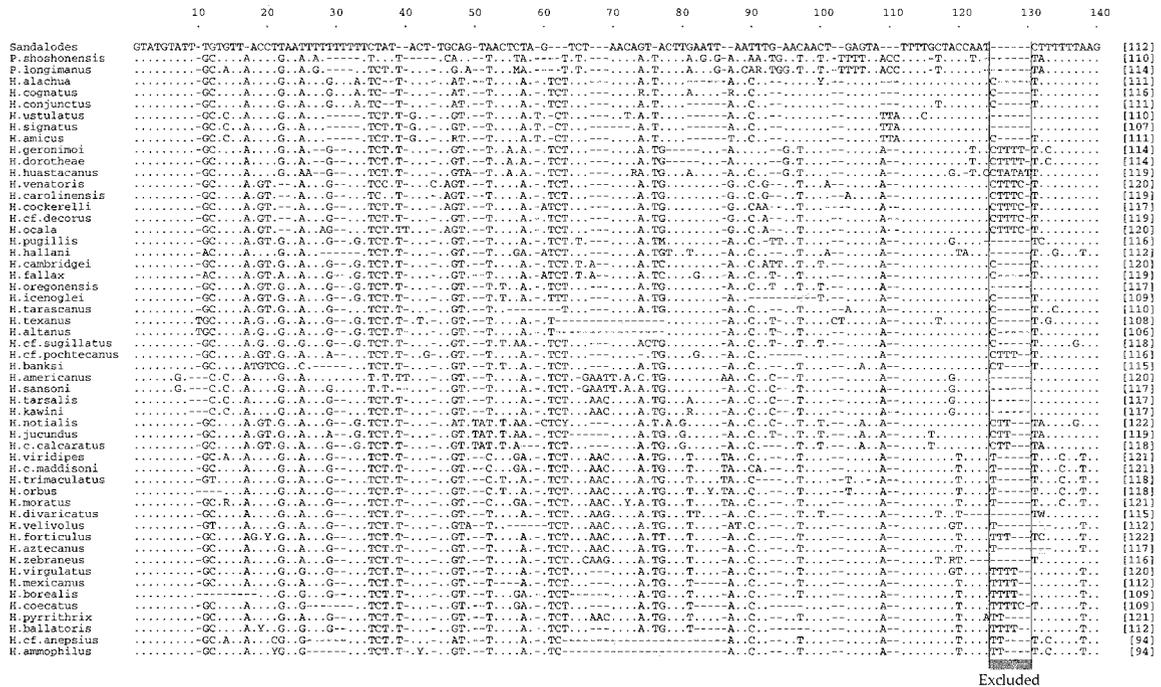
FIG. 2.—Alignment of intron I sequences. A 6-bp region at the 3′ end was excluded from all sequence analyses (see text for explanation). Shaded nucleotides, hypothesized as nonindependent indel events, were not considered in the character mapping of figure 8.

swapping, saving no more than 500 MP trees per search. Phylogenetic congruence between exon versus intron sites was assessed using the incongruence length difference (ILD) test (Farris et al. 1995; as implemented in PAUP*), considering only variable sites (Cunningham 1997). Each of 100 replicates included a heuristic search strategy as per bootstrap analyses. In all analyses considered above, multistate taxa (i.e., taxa including heterozygous sites) were scored as polymorphic, and gaps in intron sequences were treated as "fifth states."

Treating gaps in this manner is a compromise. In inspecting the EF-1α intron matrices (see figs. 2 and 3), it is clear that some indel events are phylogenetically informative (e.g., consistent with unambiguous exon signal). If we were to either exclude indel sites or treat gaps as missing, we would be excluding available phylogenetic information. Alternatively, some strings of adjacent gaps are almost certainly nonindependent but are counted as independent in a "gaps as fifth" analysis. The result is that the number of independent character changes (and bootstrap support, etc.) is potentially overestimated. We pay particular attention to this possibility in interpreting the phylogenetic results presented below.

Results

A small fraction of PCR experiments resulted in the amplification of two strong bands, one band corresponding to a ~700-bp product present in all taxa, and a smaller band at 490 bp. Analysis of a sample of genomic templates for which both bands were gel-purified and sequenced indicated that the larger product included one complete and two partial exons, separated by two introns (fig. 4). The smaller fragment corresponded to

an exon-only product (referred to below as the "intronless copy"). Alignment of Habronattus exons from both copies revealed high amino acid similarity to other arthropods, including other spiders (fig. 4). As compared with the copy with introns, the intronless copy appeared to be evolving under relaxed functional constraints, as evidenced by single-nucleotide deletions (ultimately resulting in stop codons) and relatively high rates of amino acid evolution (figs. 4 and 5). This variability contrasted strongly with extreme amino acid conservation in sequences that contained introns (see below).

The remainder of the results and analyses presented consider only those EF-1α sequences that contain introns. Such sequences were gathered for 50 Habronattus species plus 3 outgroup taxa (see table 1 for GenBank accession numbers). Available evidence suggests that these sequences correspond to an orthologous, apparently functional gene copy across the taxa surveyed. All sequences included exon data coding for 159 amino acids. There were no amino acid substitutions observed across the Habronattus-plus-Pellenes matrix, and there were only three physiochemically similar changes distinguishing the Sandalodes sequence (Thr<>Gly, Thr<>Ser, Leu<>Ileu; fig. 4). Exon nucleotide variation was less conserved, with 78, 6, and 1 variable sites at third, first, and second codon positions, respectively. The two introns that intervened in the exon sequences both appeared functional, possessing 5′ (GTAWGT) and 3′ (WAG) splice site signal sequences (fig. 4) consistent with proposed Metazoan consensus signal sequences (Senapathy, Shapiro, and Harris 1990; Mount et al. 1992). Although raw intron sequences varied in length (table 2), both sets of sequences were readily aligned
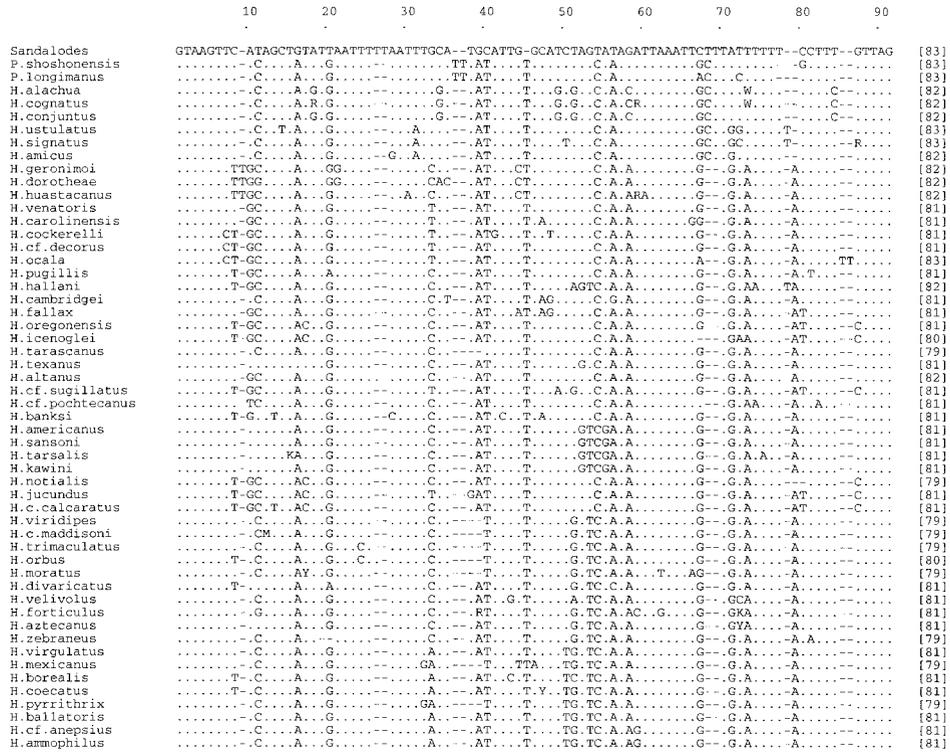
FIG. 3.—Alignment of intron II sequences. Shaded nucleotides, hypothesized as nonindependent indel events, were not considered in the character mapping of figure 8.
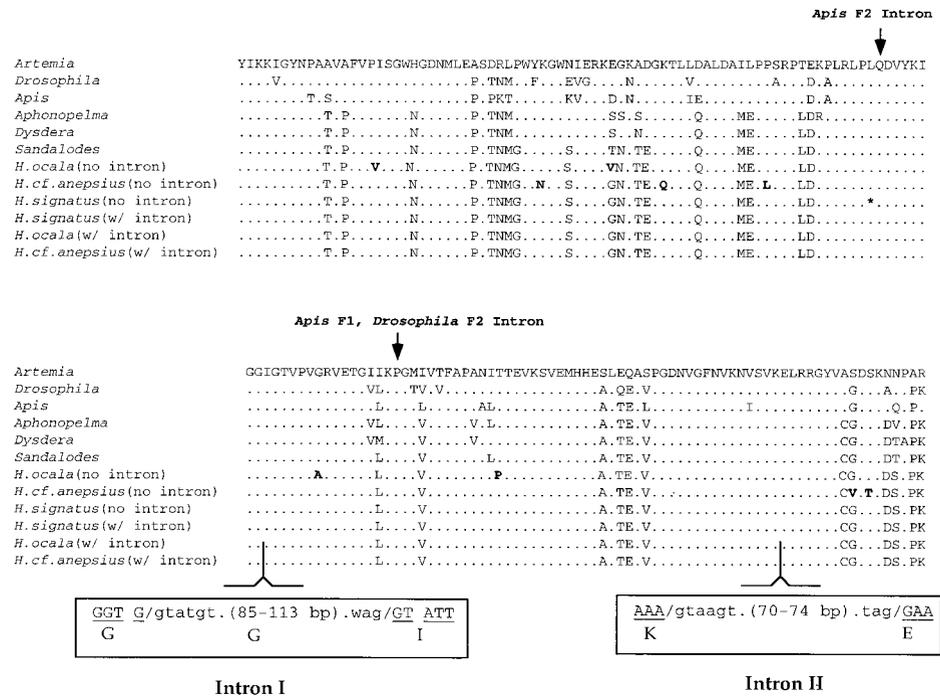

FIG. 4.—Alignment of EF-1α amino acids. Data are from Artemia (Lenstra et al. 1986), Apis (Walldorf and Hovemann 1990), Drosophila (Hovemann et al. 1988), and the spiders Aphonopelma, Dysdera (Regier and Shultz 1997), Sandalodes, and Habronattus (including sequences both with and without introns). The first amino acid corresponds to bp 1120 of Artemia (Lenstra et al. 1986). Amino acid residues in bold are unique to Habronattus sequences lacking introns; the asterisk represents a single-base frameshift deletion, resulting in a stop codon 22 codons 3′ of the deletion. Intron positions and signal sequences at exon/intron junctions in Habronattus are boxed in lower panels; intron sequences are in lowercase. Apis and Drosophila intron positions are from Danforth and Ji (1998). Habronattus and Sandalodes sequences were translated using the universal genetic code of MacClade, version 4 (Maddison and Maddison 2000).
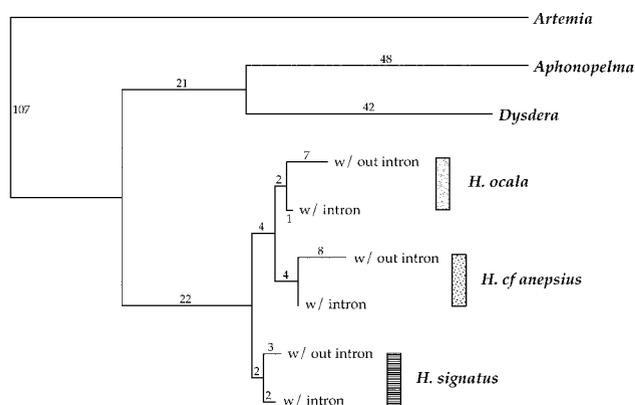
FIG. 5.—Phylogeny resulting from exhaustive parsimony analysis of EF-1α exon nucleotides, including *Habronattus* sequences with and without introns. Branch lengths are drawn proportional to the number of reconstructed parsimony changes.

manually (see figs. 2 and 3). A large fraction of the aligned intron sites were variable (193) and parsimony-informative (147). Both exon third positions and introns were AT-rich, and there was no significant variation in base composition across taxa for either intron or exon data (PAUP* chi-square analyses). Additional sequence characteristics are shown in table 2 and further discussed below.

Phylogenetic congruence between variable sites of the exon and intron sequences was assessed using the ILD test (Farris et al. 1995). This test suggested that the data were congruent ($P = 0.38$), justifying combined phylogenetic analyses. Heuristic parsimony analyses of the combined data resulted in many thousands of MP trees ($N = 14,207$, searches swapping to completion). Despite this large number of MP trees, a strict-consensus tree was reasonably well resolved (fig. 6), with many clades found in a high proportion of bootstrap replicates (e.g., more than 80% of replicates). Monophyly of species groups united by multiple morphological synapomorphies (i.e., the *agilis, amicus, americanus,* and *dorotheae* groups; see fig. 1) was strongly supported by the EF-1α data. The *coecatus, viridipes,* and *decorus* groups were never recovered as monophyletic in MP trees, although *coecatus* group monophyly could not be convincingly rejected by the data (see below). Most "straggler" taxa did not fall into obvious groups,

aside from some well-supported sister relationships (e.g., *Habronattus texanus* plus *Habronattus altanus*). An unexpected result was the placement of the Pellenes clade within Habronattus, arising near the deep-diverging *amicus* and *agilis* species groups.

Discussion
Multiple Copies

The presence of multiple copies of EF-1α in Habronattus is consistent with similar findings in other arthropod taxa. Danforth and Ji (1998) provide evidence for two EF-1α copies in the genus Apis, with each copy containing multiple introns that vary in position. Similarly, two copies (with and without introns) have been found in *Drosophila melanogaster* (Hovemann et al. 1988). Although some intron insertion positions are shared between Drosophila and Apis (Danforth and Ji 1998), the insertion positions of the Habronattus introns do not overlap with those reported for either insect (fig. 4), arguing against a shared ancient duplication. Instead, it is almost certainly the case that duplication events have occurred independently in these divergent arthropod lineages, consistent with the idea that such events have occurred multiple times independently within insects (Danforth and Ji 1998).

Although both paralogous copies appear to be functional in Drosophila and Apis, the limited amount of data available for the intronless copy suggests that this is not the case for Habronattus. One of the intronless copies includes a single nucleotide deletion that ultimately results in a stop codon, and all copies are characterized by relatively high rates of amino acid substitution as compared with copies with introns (figs. 4 and 5). This evidence for relaxed functional constraints suggests that the copy without introns may be evolving as a pseudogene. Alternatively, the copy with introns appears functional. The relative proportion of nucleotide changes at first and second versus third codon positions is as expected (table 2), and amino acid variation is nonexistent in these sequences. In addition, the intervening introns are within the range of expected sizes, include functional 5′ and 3′ splice site sequences, and are higher in AT content than adjacent exons (see Mount et al. 1992).

Table 2
Data Characteristics

Data Partition	Sequence Length (raw/aligned)	Average Base Frequencies (A, C, G, T)	No. of Var. Sites[a]	No. of PI Sites[b]	Average CI[c]
Exons					
Overall	477 bp/same	—	85	44	0.70
POS 1.	—	0.3143, 0.1700, 0.3584, 0.1573	6	1	—
POS 2.	—	0.3082, 0.2391, 0.1823, 0.2704	1	0	—
POS 3.	—	0.2923, 0.1255, 0.1522, 0.4300	78	43	—
Intron I	94–122/140	0.2800, 0.1305, 0.1354, 0.454	116	101	0.55
Intron II.	79–83/91	0.3097, 0.1152, 0.1457, 0.4295	67	46	0.66

[a] Number of variable sites, calculated assuming intron gaps as "fifth states."

[b] Parsimony-informative sites, calculated assuming intron gaps as "fifth states."

[c] Average consistency index, calculated over a randomly chosen most-parsimonious tree for combined data. Calculated assuming intron gaps as "fifth states."
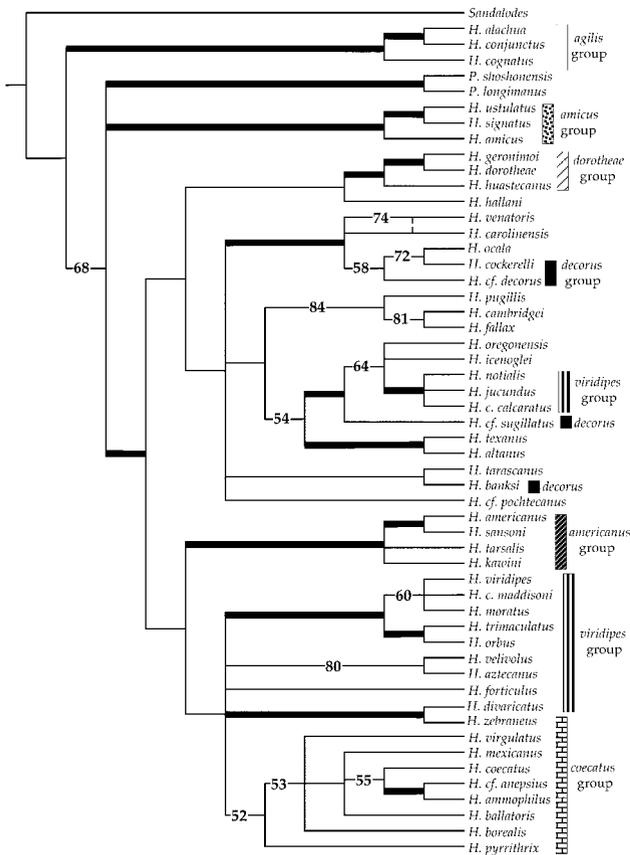
FIG. 6.—Strict consensus of most-parsimonious trees (N = 14,027; parsimony length = 727) based on analysis of combined exon and intron data. Bootstrap proportion values greater than 90% are indicated by thickened branches; all other bootstrap values greater than 50% are indicated by numbers on branches. Morphological species groups are highlighted.

FIG. 7.—Single representative parsimony phylogram from phylogenetic analysis of all exon data, including exons lacking introns (N = 4,448; parsimony length = 174). Constraint trees were used to filter all most-parsimonious (MP) trees, looking for trees in which "with intron" and "intronless" exons from the same species were sister taxa for each of the three possible sister pairs. No such trees exist in the MP tree profile.

The distribution and phylogenetic affinities of multiple EF-1α copies sampled from the same Habronattus species (see fig. 5) might be explained in one of two ways. First, it might be that derived, independent duplication events account for the relationship of copies within species being closer than that between species. We view this possibility as unlikely for the following reasons. We have detected smaller PCR products, corresponding in size to the intronless copy, in many other species and species groups of Habronattus. Although we have not sequenced these products, we believe they are homologous to the intronless copy. Also, we note that although multiple bands are not apparent in all PCR experiments, this is likely due to primer mismatch or competition rather than to copy absence. Both sets of observations are consistent with a broader phylogenetic distribution of multiple EF-1α copies in Habronattus.

This broader phylogenetic distribution is most parsimoniously explained by a common duplication at the base of Habronattus (i.e., the presence of paralogous gene copies is shared among all Habronattus), with concerted evolution maintaining high copy similarity within species. A more comprehensive perspective on this question, provided via phylogenetic analysis of all exon sequences (i.e., the three intronless copies plus all oth-

ers), suggests that the rate of this concerted evolution approximately keeps pace with the rate of speciation in the genus (fig. 7). Based on functional considerations, we hypothesize that the directionality of concerted evolution is from "with intron" to "intronless" at a rate high enough to promote concerted evolution but perhaps not high enough to maintain the functionality of the intronless copy. Future studies directed at testing this hypothesis should include not only more intronless sequences, but also sequences from sets of more closely related Habronattus, thus providing finer resolution of rate dynamic issues.

Intron/Exon Dynamics and Utility

To date, most phylogenetic studies incorporating EF-1α have relied on exon variation and, accordingly, have considered systematic problems that are generally deeper than those considered here (e.g., among genera or tribes). Reported levels of exon variation within genera indicate limited divergence (e.g., up to 1% within moths; Cho et al. 1995), with essentially all nucleotide variation occurring at third-position sites. Mitchell, Mitter, and Regier (2000) estimate likelihood-based relative rates of 4:1:81 at first : second : third codon positions, re-

Table 3
R Matrix Estimates

	TRANSITIONS		TRANSVERSIONS			
	C<>T	A<>G	A<>T	A<>C	C<>G	G<>T
Exon	26.44	12.88	5.68	1.19	1.42	1
Intron . . .	2.57	2.94	0.46	0.95	2.27	1

NOTE.—Likelihood rate parameters were estimated over a randomly chosen most-parsimonious tree assuming a GTR+Γ model. The following taxa were excluded from consideration for all estimates: *Pellenes shoshonensis, Habronattus icenoglei, Habronattus tarascanus, Habronattus altanus, Habronattus texanus, Habronattus divaricatus, Habronattus velivolus, Habronattus zebraneus, Habronattus mexicanus, Habronattus borealis, Habronattus ballatoris,* and *Habronattus* cf. *anepsius.* These sequences include long gaps in intro 1 (see fig. 2). When these sequences are excluded, the number of intron sites considered increases from 115 to 139 (of 231 total aligned sites).

spectively. Likelihood parameter estimates for the Habronattus exon data suggest similar patterns (to facilitate comparison with intron data [see below], some taxa were excluded from likelihood analyses; estimates using the full exon matrix are very similar). Using a best-fit GTR+Γ maximum-likelihood model (model fit assessed using MODELTEST; Posada and Crandall 1998), relative-rate parameters estimated over a randomly chosen MP tree correspond roughly to 6:1:112 at first : second : third codon positions, respectively. A low estimated value of the gamma distribution shape parameter over all sites indicates a distribution with a strong left skew (α = 0.14), reflecting a combination of many invariant sites with fewer, more variable, sites (see fig. 13 of Swofford et al. 1996). These variable sites appear to be evolving at approximately the same rate, given that the GTR+Γ model explains the data as well as both the GTR+%I and the GTR+%I+Γ models. Most exon site variation is accounted for by transitions (see table 3).

In considering both intron and exon data, are we simply adding additional sites that are evolving approximately like third position exon sites, or are we adding sites with substantively different evolutionary dynamics? To address this question, we independently estimated a best-fit likelihood model for the intron data. As for exon sites, this corresponds to a GTR+Γ model. Because a large fraction of intron I aligned sites include at least one indel in the full taxon matrix (and thus are ignored in likelihood estimates), we increased the total number of sites considered by excluding some gap-rich sequences (see table 3). The estimated R matrix for intron sites differs markedly from that estimated for exon sites (table 3). The transition bias is much less extreme, with some transversion rates (C<>G) approximating that of transitions. A higher estimated value of the gamma distribution shape parameter indicates greater symmetry (α = 3.09), reflecting more equal rates across all sites. Comparison of relative rates of evolution suggests that, as a site class, introns are evolving about twice as fast as exon third positions (relative-rate ratio = 2.1:1).

The differences in rate and substitution dynamics in EF-1α introns versus exons suggest the possibility that these sequence partitions may contribute in different, and perhaps complementary, ways to phylogenetic resolution in Habronattus. For example, we might ex-
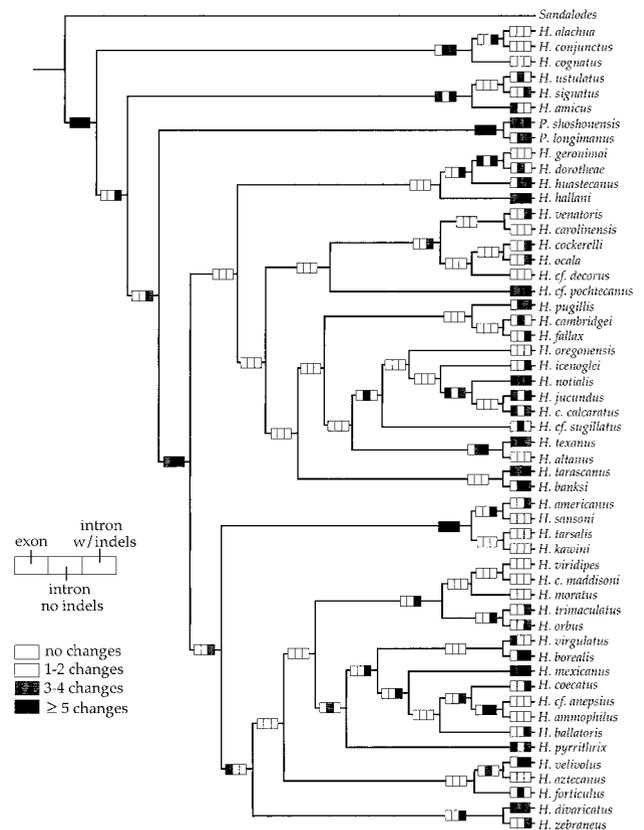
FIG. 8.—Character mapping of nucleotide and indel changes for intron and exon data, calculated using the "trace all changes" option of MacClade, version 4. "All possible" character changes were optimized over a random dichotomous resolution of a randomly chosen most-parsimonious tree, with the nucleotide data recoded as "standard" and gaps recoded as state "4." Changes are partitioned into three categories, including exon sites, intron sites without indels, and intron sites with indels, with the amount of change distinguished by relative shading. Although a fourth category is possible (i.e., changes from nucleotide to nucleotide at intron sites with indels), such changes were included in the intron-sites-with-indels site class. A small number of hypothesized nonindependent changes in the intron-sites-with-indels class were not mapped (see figs. 2 and 3).

pect the more rapidly evolving introns to provide more phylogenetic signal at shallow levels, complemented by deeper signal contributed by exons. Intron sites that include gaps may potentially confound such an interpretation, as such sites make up a large fraction of intron sites, and we cannot be sure that these sites are evolving like the "typical" intron site (i.e., they may be evolving more slowly). To address this issue, and to look for complementary phylogenetic signal at different divergence depths, we reconstructed changes of three separate site classes (i.e., exon sites, intron sites without gaps, and intron sites with at least one gap) on a randomly chosen MP tree (fig. 8). A visual scan of the two right-hand boxes on each branch in figure 8 shows that of 93 branches showing change in either intron site class, 70 boxes remain the same or get darker as one moves to the right, whereas only 23 boxes get lighter. This would support the general conclusion that as a site class, the "with gaps" class changes as much as or more than the "without gaps" class (note that the reconstructed chang-

es in the ''with gaps'' class are not all nucleotide <>
indel changes; see fig. 8 legend). Patterns of intron :
exon complementary resolution are less obvious. Some
species groups show a pattern in which most exon
changes occur basally, with internal resolution coming
from introns (e.g., the *americanus* group), but, in gen-
eral, a simple ''exon deep, intron shallow'' dynamic
does not adequately explain the Habronattus data.

Congruence with Prior Phylogenetic Hypotheses

Some Habronattus species groups are supported by
enough character evidence to perhaps be considered as
areas of ''known phylogeny.'' Examples include the *do-
rotheae, americanus, amicus,* and *agilis* groups, defined
not only by multiple morphological synapomorphies
(see fig. 1; Griswold 1987), but also by other characters
not formally analyzed in a phylogenetic context (e.g.,
agilis group characters discussed in Maddison and Strat-
ton [1988]). These four clades are recovered and strong-
ly supported by the EF-1α data (see fig. 6). At least
some character support comes from exon and intron
sites without gaps (fig. 8), although this pattern is less
obvious for the *dorotheae* group. Here, intron sites with
gaps provide most group support (fig. 8), but visual in-
spection of intron matrices does not indicate that such
support is obviously overestimated by strings of non-
independent gaps (see figs. 2 and 3).

Aside from the recovery of well-established
groups, what do the EF-1α data suggest that is unex-
pected and potentially interesting? One well-supported,
perhaps cryptic, species group is composed of a subset
of ''*decorus*'' group taxa (*Habronattus cockerelli* and
Habronattus cf. *decorus*) allied with a trio of species
(*Habronattus venatoris, Habronattus carolinensis,* and
Habronattus ocala) also previously thought to be related
(see fig. 1; Griswold 1987). This clade is recovered in
a high proportion of bootstrap analyses (fig. 6) and is
supported by all three classes of character change (fig.
8). Sequences of other members of the ''*decorus* group''
(*Habronattus banksi* and *Habronattus* cf. *sugillatus*) are
never placed with the above taxa, suggesting that this
species group may not be a natural group. This would
not be surprising, as the morphological support for this
hypothesized clade is tenuous, based on the absence of
three correlated characters (ventral fringe on femur, pa-
tella, and tibia I) that are homoplasious elsewhere in the
genus (Griswold 1987).

The species-rich *viridipes* and *coecatus* groups in-
clude taxa with males possessing striking first- and
third-leg modifications used in courtship display. If
Griswold's (1987) phylogeny is correct, it would suggest
that third-leg ornaments have arisen only once in Ha-
bronattus, with the absence of such modifications in *Ha-
bronattus moratus, Habronattus orbus,* and *Habronattus
trimaculatus* (plus other species not sampled) represent-
ing cases of secondary loss (fig. 1). In this case, the EF-
1α data present a mixture of ambivalence, novel confir-
mation, and unexpected placements. Sequences from
members of the *coecatus* group are allied with those of
the *viridipes* group in MP trees, but because of the

placement of *Habronattus zebraneus* with *Habronattus
divaricatus,* the *coccatus* group itself is not monophy-
letic. This latter sister relationship may be an artifact of
treating gaps as fifth states (see figs. 2 and 8), and trees
including a monophyletic *coecatus* group are only six
steps longer than MP (733 vs. 727). The DNA data ap-
pear to confirm Griswold's (1987) hypothesis regarding
ornament loss in *H. moratus, H. orbus,* and *H. trima-
culatus,* as these species are placed with otherwise mor-
phologically similar taxa in a well-supported clade (fig.
6). One of the most surprising results of this study is
the placement of three *viridipes* group sequences (*Ha-
bronattus notialis, Habronattus jucundus,* and *Habron-
attus calcaratus calcaratus*) into a very distinctive clade
far separated from other group members. These taxa are
very similar to *Habronattus viridipes* and *Habronattus
calcaratus maddisoni* (*H. calcaratus calcaratus* is clas-
sified as a member of the same species!), sharing several
morphological, ecological, and behavioral characteris-
tics (Griswold 1987; unpublished data). Their placement
in the EF-1α tree might indicate a potential orthology/
paralogy problem, but intriguingly, mitochondrial DNA
sequences indicate the same separation for the same taxa
(unpublished data).

Phylogenetic relationships among species groups of
Habronattus, particularly deep in the tree, are currently
ambiguous (see fig. 1). The EF-1α data suggest some
potential resolution of this ambiguity, indicating that the
agilis and *amicus* groups are basal lineages in the genus,
with a well-supported branch separating these groups
from more derived Habronattus (fig. 6). The character
support for this separation comes from all three site clas-
ses (fig. 8). Relationships among these basal groups, in-
cluding species currently placed in the genus Pellenes,
remain ambiguous. Pellenes is embedded within Ha-
bronattus in MP trees (fig. 6), but character support for
these deep relationships is weak and based in large part
on intron sites with gaps (see fig. 8). Given the strong
morphological evidence for Habronattus monophyly
(Griswold 1987), we would be surprised if Pellenes were
truly nested within Habronattus.

Habronattus is a large and complex genus, with over
90 species described and perhaps half that number wait-
ing to be described. Fully understanding this diversity from a
phylogenetic perspective is a nontrivial task and will ul-
timately require multiple lines of character evidence for a
large number of species. Given the generally favorable re-
sults presented here, we feel that EF-1α sequence data
should represent one such line of character evidence.
Whether or not EF-1α will behave similarly in other spider
taxa requires more study, but we believe that the gene has
clear potential, particularly given the generally positive re-
sults coming from studies of other arthropod groups.

LITERATURE CITED

Bond, J. E., M. C. Hedin, M. G. Ramirez, and B. D. Opell. 2001. Deep molecular divergence in the absence of morphological and ecological change in the Californian coastal dune endemic trapdoor spider Aptostichus simus. Mol. Ecol. **10**:899–910.

Caterino, M. S., S. Cho, and F. A. H. Sperling. 2000. The current state of insect molecular systematics: a thriving Tower of Babel. Annu. Rev. Ecol. Syst. **45**:1–54.

Cho, S., A. Mitchell, J. C. Regier, C. Mitter, R. W. Poole, T. P. Friedlander, and S. Zhao. 1995. A highly conserved nuclear gene for low-level phylogenetics: Elongation factor–1α recovers morphology-based tree for Heliothine moths. Mol. Biol. Evol. **12**:650–656.

Coddington, J. A., and H. W. Levi. 1991. Systematics and evolution of spiders (Araneae). Annu. Rev. Ecol. Syst. **22**:565–592.

Cunningham, C. W. 1997. Can three incongruence tests predict when data should be combined? Mol. Biol. Evol. **14**:733–740.

Danforth, B. N., and S. Ji. 1998. Elongation factor–1α occurs as two copies in bees: implications for phylogenetic analysis of EF-1α sequences in insects. Mol. Biol. Evol. **15**:225–235.

Danforth, B. N., H. Sauquet, and L. Packer. 1999. Phylogeny of the bee genus Halictus (Hymenoptera: Halictidae) based on parsimony and likelihood analyses of nuclear EF-1α sequence data. Mol. Phylogenet. Evol. **13**:605–618.

Farris, J. S., M. Kallersjo, A. G. Kluge, and C. Bult. 1995. Testing significance of incongruence. Cladistics **10**:315–319.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.

Gillespie, R. G., H. B. Croom, and G. L. Hasty. 1997. Phylogenetic relationships and adaptive shifts among major clades of Tetragnatha spiders (Araneae: Tetragnathidae) in Hawai'i. Pacific Sci. **51**:380–394.

Griswold, C. E. 1987. A revision of the jumping spider genus Habronattus F. O.P.-Cambridge (Araneae; Salticidae), with phenetic and cladistic analyses. Univ. Calif. Publ. Entomol. **107**:1–345.

Griswold, C. E., J. A. Coddington, N. I. Platnick, and R. R. Forster. 1999. Towards a phylogeny of Entelegyne spiders (Araneae, Araneomorphae, Entelegynae). J. Arachnol. **27**:53–63.

Hedin, M. C. 2001. Molecular insights into species phylogeny, biogeography, and morphological stasis in the ancient spider genus Hypochilus (Araneae, Hypochilidae). Mol. Phylogent. Evol. **18**:238–251.

Hovemann, B., S. Richter, U. Walldorf, and C. Ciepluch. 1988. Two genes encode related cytoplasmic elongation factors 1α (EF-1α) in Drosophila melanogaster with continuous and stage specific expression. Nucleic Acids Res. **16**:3175–3194.

Lenstra, J. A., A. van Vliet, A. C. Arnberg, F. J. van Hemert, and W. Moller. 1986. Genes coding from the elongation factor EF-1α in Artemia. Eur. J. Biochem. **155**:475–483.

Maddison, D. R., and W. P. Maddison. 2000. MacClade 4: analysis of phylogeny and character evolution. Version 4.0. Sinauer, Sunderland, Mass.

Maddison, W. P. 1982. XXXY sex chromosomes in males of the jumping spider genus Pellenes (Araneae: Salticidae). Chromosoma **85**:23–37.

———. 1997. Gene trees in species trees. Syst. Biol. **46**:523–536.

Maddison, W. P., and M. McMahon. 2000. Divergence and reticulation among montane populations of a jumping spider (Habronattus pugillis Griswold). Syst. Biol. **49**:400–421.

Maddison, W. P., and G. E. Stratton. 1988. Sound production and associated morphology in male jumping spiders of the Habronattus agilis species group (Araneae, Salticidae). J. Arachnol. **16**:199–211.

Masta, S. E. 2000. Phylogeography of the jumping spider Habronattus pugillis (Araneae: Salticidae): recent vicariance of sky island populations? Evolution **54**:1699–1711.

Mitchell, A., C. Mitter, and J. C. Regier. 2000. More taxa or more characters revisited: combining data from nuclear protein-coding genes for phylogenetic analyses of Noctuoidea (Insecta: Lepidoptera). Syst. Biol. **49**:202–224.

Mount, S. M., C. Burks, G. Hertz, G. D. Stormo, O. White, and C. Fields. 1992. Splicing signals in Drosophila: intron size, information content, and consensus sequences. Nucleic Acids Res. **20**:4255–4262.

Peckham, G. W., and E. G. Peckham. 1889. Observations on sexual selection in spiders of the family Attidae. Occas. Pap. Wis. Nat. Hist. Soc. **1**:3–60.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics **14**:817–818.

Reed, R. D., and F. A. H. Sperling. 1999. Interaction of process partitions in phylogenetic analysis: an example from the swallowtail genus Papilio. Mol. Biol. Evol. **16**:286–297.

Regier, J. C., and J. W. Shultz. 1997. Molecular phylogeny of the major arthropod groups indicates polyphyly of crustaceans and a new hypothesis for the origin of hexapods. Mol. Biol. Evol. **14**:902–913.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shultz, J. W., and J. C. Regier. 2000. Phylogenetic analysis of arthropods using two nuclear protein-encoding genes supports a crustacean + hexapod clade. Proc. R. Soc. Biol. Sci. B **267**:1011–1019.

Senapathy, P., M. B. Shapiro, and N. L. Harris. 1990. Splice junctions, branch point sites, and exons: sequence statistics, identification, and application to genome project. Methods Enzymol. **183**:252–278.

Shahjahan, R. M., K. J. Hughes, R. A. Leopold, and J. D. DeVault. 1995. Lower incubation temperature increases yield of insect genomic DNA isolated by the CTAB method. Biotechniques **19**:333–334.

Swofford, D. L. 1999. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer, Sunderland, Mass.

Swofford, D. L., G. J. Olsen, P. J. Waddell, and D. M. Hillis. 1996. Phylogenetic inference. Pp. 407–514 in D. M. Hillis, C. Moritz, and B. K. Mable, eds. Molecular systematics. 2nd edition. Sinauer, Sunderland, Mass.

Walldorf, U., and B. T. Hovemann. 1990. Apis mellifera cytoplasmic elongation factor 1α (EF-1α) is closely related to Drosophila melanogaster EF-1α. FEBS **267**:245–249.

Zehethofer, K., and K. Sturmbauer. 1998. Phylogenetic relationships of central European wolf spiders (Araneae: Lycosidae) inferred from 12S ribosomal DNA sequences. Mol. Phylogenet. Evol. **10**:391–398.

```
                    10        20        30        40        50        60        70        80        90       100       110       120       130       140
                     .         .         .         .         .         .         .         .         .         .         .         .         .         .

Sandalodes        GTATGTATT-TGTGTT-ACCTTAATTTTTTTTTTTCTAT--ACT-TGCAG-TAACTCTA-G---TCT---AACAGT-ACTTGAATT--AATTTG-AACAACT--GAGTA---TTTTGCTACCAAT------CTTTTTTAAG  [112]
P.shoshonensis    .........-GC....A...G..A.A.-----.T...T-----.CA.--T...TA.----T.T.-------.A.T....A.G.G-A..AA.TG..T..T.-TTTT..ACC....-T....T.------TA........  [110]
P.longimanus      .........-GC.A...A...G..A.G.-----.TCT.T-....G.A.--T...MA.----T.T.-------.A.T....A...G-A.CAR.TGG.T..T.-TTTT..ACC....-T......------TA........  [114]
H.alachua         .........-GC....A...G..A...G...A.TC--T-.....AT.--T....A.-.TCT.........A.T.....-------A..C.-.......Y.--------C-----T.......  [111]
H.cognatus        .........-GC....A...G..A...G...A.TC--T-.....AT.--T....A.-.TCT........R.T....A....-R..C.-.......-........C-----T.......  [116]
H.conjunctus      .........-GC....A...G..A...G...A.TC--T-.....AT.--T....A.-.TCT.........A.T.........-A..C.-.......--.......T......C-----T.......  [111]
H.ustulatus       .........-GC.C..A....G..A.........----TCT.T-G..-..GT.--T....A.T.-CT........-....T.A.T.........-A..C.-------...TTA...C.......------T.......  [110]
H.signatus        .........-GC.C..A....G..A.........----TCT.T-G..-..GT.--T....A.T.-CT........-..A.T.........-A..C.-------...TTA..........T.------T.......  [107]
H.amicus          .........-GC.C..A....G..A.........----TCT.T-G..-..RT.--T....A.T.-CT........-....A.T.........-T..C.-------...TTA..........C-----T.......  [111]
H.geronimoi       .........-GC....A...G..A.--G-...TCT.T-.....GT.--T..A.A.-.TCT.........A.TG------....-A.....--G.T............A--..........T..CTTTT-T.C.......  [114]
H.dorotheae       .........-GC....A...G..A.-...TCT.T-.....GT.--T..A.e.a.-.TCT.........A.TG------....-A.....--G.T............A--..........T..CTTTT-T.C.......  [114]
H.huastacanus     .........-GC....A...G..AA--G-...TCT.T-.....GTA--T..A.A.-.TCT........RA.TG...A.--..A..C.--G.T.........A--.....G.-T.CCTATATT  [119]
H.venatoris       .........-GC....A.GT.---A...G-...TCC.T-...C.AGT.--T....A.-.TCT.........A.TG.........-G..C.G---.T..A-......A--........CTTTC-T.......  [120]
H.carolinensis    .........-GC....A.GT.---A...G-...TC..T-....AGT.--T....A.-.TCT.........A.TG.........-G..C.G--.T...--A...A--........CTTTC-T.......  [119]
H.cockerelli      .........-GC....A.GT.---A...G-...TCT.T-....AGT.--T....A.-ATCT.........A.TG.........-G..CAA--.T.........A--........CTTTC-T.......  [117]
H.cf.decorus      .........-GC....A.GT.---A...G-...TCT.T-....GT.--T....A.-.TCT.........A.TG.........-G..C.A---.T.........A--........CTTTC-T.......  [119]
H.ocala           .........-GC....A.GT.---A.-AG-...TC.TT..-..AGT.--T....A.-.TCT.........A.TG.........-G..C.A---.T.........A--........CTTTC-T.......  [120]
H.pugillis        .........-GC....A.GT.G.A...G--..G.TCT.T-.....GT.--T....A.-.TCT.T.------A.TM.........-A..C.-TT..T.........A--........G.....------TC.......  [116]
H.hallani         .........-AC....A....G..A.-------TCT.T-.....GT.--T...GA.-ATCT.........A.TGT..T...-A..C..-...T....A--........A--........TA...------T..G...T..  [112]
H.cambridgei      .........-GC....A.GT.G..A...G--..G.TCT.T-.....GT.--T....A.-.TC.T.A-......A.TC.......-A..C.ATT..T..T.........A--........C-----T.......  [120]
H.fallax          .........-AC....A.GT.A..A...G--..G.TCT.T-.....GT.--T....A.-ATCT.T.A-......A.TC.....G....A..C.-T-..T.........A--........C-----T.......  [119]
H.oregonensis     .........-GC....A.GT.G..A...G--..G.TCT.T-.....GT.--T..T..A.-.TCT.........A.TG.........-A..C.T-..T..T.........A--........-----T.......  [117]
H.icenoglei       .........-GC....A.GT.G..A...G--..G.TCT.T-.....GT.--T..T..A.-.TTT........-A.TG.........-A........---T......A--........C-----T.......  [109]
H.tarascanus      .........-GC....A.GT.G..A...G--...TCT.T-.....GT.--T.-...----...-T.......A.TG.........-A..C.-...T....--A..A--........C-----T..C.......  [110]
H.texanus         ........TGC....A.G..G..A...G--..G.TCT.T-.T-...GT.--T....A.-.T--------........-A..C.T-..T....CT.....A--........T.....C-----T.G.......  [108]
H.altanus         ........TGC....A.G..G..A...G--..G.TCT.T-.....GT.--T....A.-.T--------........-A..C.T-..T.........A--........C-----T.......  [106]
H.cf.sugillatus   .........-GC....A.G..G..G...G--..G.TCT.T-.....GT.--T..T.AA.-.TCT.........ACTG........-A..C.T-..T..T.........A--........C-----T.....G...  [118]
H.cf.pochtecanus  .........-GC....A.GT.G..A...A--...TCT.T-..G-...GT.--T....A.-.TCT.........---.TG....G....-A..C.------........A--........CTTTT--T.......  [116]
H.banksi          .........-GC....ATGTCG...C.-------TCT.T-.....GT.--T..T.A.-.TCT.........-A.TG........-A..C.-...T....--A.A.A--........CT---T.......  [115]
H.americanus      ......G.---C.C..A....G..A.........T.T.TT.....GT.--T....A.-.TCT.-GAATT.A.C.TG.........AA..C.C--.T........-A........G....-------------  [120]
H.sansoni         ......G.---C.C..A....G..A.........T.T.------.GT.--T....A.-.TCT.-GAATT.A.A.TG........-A..C.C--.T........-A........G....-------------  [117]
H.tarsalis        .........---C.C..A....G..A...G--...TCT.T-.....GT.--T....A.-.TCT..AAC....A.TG...A.-.TCT.-......A........-A........G....-------------  [117]
H.kawini          .........-C.C..A....G..A...G--...TCT.T-.....GT.--T....A.-.TCT..AAC....A.TG...R...-A..C.C--.T........-A........G....-------------  [117]
H.notialis        .........-GC....A.GT.G..A...G--..G.TCT.T-.....AT.TAT.TAA.-CTCY........-........A.T.A.G.....-A..C.C-..T..T....-.A..A--........CTT---TA....G...  [122]
H.jucundus        .........-GC....A.GT.G..A...A--..G.TCT.T-.....GT.TAT.TAA.-.TCT-------........A.TG..G.....-A..C.T-..T..T....-.A..A--........T........CTT---TA........  [119]
H.c.calcaratus    .........-GC....A.GT.G..A...G--..G.TCT.T-.....GT.TAT.TA----.TCT.........-A.TG..G.....-A..C.T-..T....-.A..A--..T........CTT---TA........  [118]
H.viridipes       .........-GC.A..A....G..A...G--...TCT.T-.....GT.--C...GA.-.TCT..AAC....A.TG...T....TA.C.-...T.........A--........T...T-----T...C..T..  [121]
H.c.maddisoni     .........-GC....A....G..A...G--...TCT.T-.....GT.--C...GA.-.TCT..AAC....A.TG...T....TA.CA.-...T.........A--........T...T-----T...C..T..  [121]
H.trimaculatus    .........-GT....A....G..A...G--...TCT.T-.....GT.--C.T..A.-.TCT..AAC....A.TG...T....TA.C.-...T...--T.-A--........T...T-----T...C..T..  [118]
H.orbus           .........-----...A....G..A...G--...TCT.T-.....GT.--C.T..A.-.TCT..AAC....A.TG...T..Y.TA.C.-...T...--T.-A--........T...T-----T...C..T..  [118]
H.moratus         .........-GC.R..A....G..A...G--...TCT.T-.....GT.--C...GA.-.TCT..AAC..Y.A.TG...T...TA.C.-...T.........A--........T...T-----T...C..T..  [121]
H.divaricatus     .........-GC....A....G..A...G--...TCT.T-.....GT.--T...A.-.TCT..AAG....A.TG...A..TT.-.A..C.-...T.T......A--......T..T..T---------TW......  [115]
H.velivolus       .........-GT....A....G..A...G--...TCT.T-.....GTA--T.------TCT..AAC....A.TG...T...-AT.C.-...T.........A--........GT...T-----..-.....T..  [112]
H.forticulus      .........-GC....AG.Y.G..A...G--...TCT.T-.....GT.--T....A.-.TCT..AAC....A.TT..T...-A..C.-...T.........A--........T...TTT---TC....T..  [122]
H.aztecanus       .........-GC....A....G..A...G--...TCT.T-.....GT.--T....A.-.TCT..AAC....A.TG...T...-A..C.-...T.........A--........T...T-----..-.....T..  [117]
H.zebraneus       .........-GC....A....G..A...G--...TCT.T-.....GT.--T....A.-.TCT..CAAG....A.TG...T...-A..C.-...T.........A--......T.RT---------T.......  [116]
H.virgulatus      .........-GC....A....G..G...G--...TCT.T-.....GT.--T..A.-.TCT.-......A.TG..T....-A..C.-...T.........A--........GT...TTTT--.....T..  [120]
H.mexicanus       .........-GC....A....G..A...G--...TCT.T-.....GT.--T----.A.-.TCT........A.TG...T----.-A..C.-..T.........A--........T...TTTT--.....T..  [112]
H.borealis        .........------------...G..G...G--...TCT.T-.....GT.--T...GA.-.TCT-------.A.TG...T..-A..C.-...T.........A--........T...TTTT--.....T..  [109]
H.coecatus        .........-GC....A....G..G--------TCT.T-.....GT.--T...GA.-.TCT........A.TG...T----.-A..C.-...T.........A--........T...TTTTC-T.....T..  [109]
H.pyrrithrix      .........-GC....A....G..A...G--...TCT.T-.....GT.--T....A.-.TCT..AAC....A.TG...T....-A..C.-...T.........A--........T..ATT---........T..  [121]
H.ballatoris      .........-GC....A.Y.G..G...G--...TCT.T-.....GT.--T...A.-.TCT........A.TG...T----.-A..C.-......A--........T...TTTT--.......T..  [112]
H.cf.anepsius     .........-GC.A..A....CG..G-------TCT.T-.....AT.--T....A.-.TC-------------------A..C.-...T.........A--........T...TT----T.C....T..  [94]
H.ammophilus      .........-GC....A...YG..G-------TCT.T-.Y.-..GT.--T....A.-.TC-------........-----------A..C.-...T.........A--......T...TT----T.C....T..  [94]

                                                                                                                                        Excluded
```

```
                          10        20        30        40        50        60        70        80        90
                           .         .         .         .         .         .         .         .         .
Sandalodes           GTAAGTTC-ATAGCTGTATTAATTTTTAATTTGCA--TGCATTG-GCATCTAGTATAGATTAAATTCTTTATTTTTT--CCTTT--GTTAG   [83]
P.shoshonensis       ........-.C....A...G.....--.........TT.AT....T........C.A.........GC.........---G....--....   [83]
P.longimanus         ........-.C....A...G.....--.........TT.AT....T........C.A.........AC...C.....---....--....   [83]
H.alachua            ........-.C....A.G.G.....--.....G.--.AT....T...G.G..C.A.C.......GC....W......--...C--.....   [82]
H.cognatus           ........-.C....A.R.G.....--.....G.--.AT....T...G.G..C.A.CR.......GC....W......--...C--.....   [82]
H.conjuntus          ........-.C....A.G.G.....--.....G.--.AT....T...G.G..C.A.C.......GC...........--...C--.....   [82]
H.ustulatus          ........-.C..T.A...G.....--...A.....--.AT....T........C.A.........GC..GG.....T-...--......   [83]
H.signatus           ........-.C....A...G.....--...A.....--.AT....T....T...C.A.........GC..GC.....T-......--R....   [83]
H.amicus             ........-.C....A...G.....--G..A.....--.AT....T........C.A.........GC..G.........--......----   [82]
H.geronimoi          .......TTGC....A...G.........--....C.....--.AT...CT........C.A.A......G--.G.A...-A............   [82]
H.dorotheae          .......TTGG....A..GG.........--..CAC--.AT...CT........C.A.A......G--.A.........-A............   [82]
H.huastacanus        .......TTGC....A...G.........--..A..C.--.AT...CT........C.A.ARA......G--.G.A...-A............   [82]
H.venatoris          ........-GC....A...G.........--....T.....--.AT....T........C.A.A......G--.G.A...-A............   [81]
H.carolinensis       ........-GC....A...G.........--....T.....--.AT...T.A......C.A.A......GG--.G.A...-A............   [81]
H.cockerelli         ......CT-GC....A...G.........--....T.....--.ATG...T..T.....C.A.A......G--.G.A...-A............   [81]
H.cf.decorus         ......CT-GC....A...G.........--....T.....--.AT....T.......C.A.A......G--.G.cf..-A.........--...   [81]
H.ocala              ......CT-GC....A...G.........--....T.....--.AT....T.......C.A.A......A--.G.A...-A.....TT.....   [83]
H.pugillis           ........T-GC....A...A.........--....C.....--.AT....T........C.A.A......G--.G.A...-A.T....--....   [81]
H.hallani            ........T-GC....A...G.........--....C.....--.AT....T....AGTC.A.A......G--.G.AA...TA............   [82]
H.cambridgei         ........-.C....A...G.........--....C.T--.AT....T.AG.....C.G.A......G--.G.A...-A............   [81]
H.fallax             ........-GC....A...G.........--....C.....--.AT...AT.AG.....C.A.A......G--.G.A...-AT....--....   [81]
H.oregonensis        ........T-GC....AC..G.........--....C.....--.AT....T......C.A.A......G--.G.A...-AT....--C....   [81]
H.icenoglei          ........T-GC....AC..G.........--....C.....--.AT....T......C.A.A......----.GAA...-AT....--C....   [80]
H.tarascanus         ........-.C....A...G.........--....C.....----.......T......C.A.A......G--.G.A...-A............   [79]
H.texanus            ........-......G.........--....C.....--.AT....T......G.C.A.A......G--.G.A...-A............   [81]
H.altanus            ........-GC....A...G.........--....C.....--.AT....T........C.A.A......G--.G.A...-A............   [82]
H.cf.sugillatus      ........T-GC....A...G.........--....T.....--.AT....T...A.G..C.A.A......G--.G.A...-AT....--C....   [81]
H.cf.pochtecanus     ........-TC....A...G.........--....C.....--.AT....T........C.A.A........--.G.AA...-A..A............   [81]
H.banksi             ........T-G..T..A...G......--C.....C.....--.AT.C..T.A.......C.A.A......G--.G.A...-A............   [81]
H.americanus         ........-......A...G.........--....C.....--.AT....T.....GTCGA.A......G--.G.A...-A............   [81]
H.sansoni            ........-......A...G.........--....C.....--.AT....T.....GTCGA.A......G--.G.A...-A............   [81]
H.tarsalis           ........-......KA...G.........--....C.....--.AT....T.....GTCGA.A......G--.G.A.A..-A............   [81]
H.kawini             ........-......A...G.........--....C.....--.AT....T.....GTCGA.A......G--.G.A...-A............   [81]
H.notialis           ........T-GC....AC..G.........--....C.....--.AT....T......C.A.A......G--.G.A...---....--C....   [79]
H.jucundus           ........T-GC....AC..G.........--....T.--GAT....T......C.A.A......G--.G.A...-AT....--C....   [81]
H.c.calcaratus       ........T-GC.T..AC..G.........--....C.....--.AT....T......C.A.A......G--.G.A...-AT....--C....   [81]
H.viridipes          ........-.C....A...G.........--....C.....----T....T.....G.TC.A.A......G--.G.A...-A............   [79]
H.c.maddisoni        ........-.CM...A...G.........--....C.....----T....T.....G.TC.A.A......G--.G.A...-A............   [79]
H.trimaculatus       ........-.C....A...G...C.....--....C.....----T....T.....G.TC.A.A......G--.G.A...-A............   [79]
H.orbus              ........T-C....A...G...C.....--....C.....----T....T.....G.TC.A.A......G--.G.A...-A............   [80]
H.moratus            ........-.C....AY..G.........--....C.....----T....T.....G.TC.A.A...T...AG--.G.A...-A............   [79]
H.divaricatus        ........T-......A...A.........--....C.....--.AT....T.....G.TC.C.A......G--.G.A...-A............   [81]
H.velivolus          ........-.C....A...G.........--....C.....--.AT..G.T.....A.TC.A.A......G--.GCA...-A............   [81]
H.forticulus         ........-.G....A...G.........--....C.....--.RT....T.....G.TC.A.AC..G.....G--.GKA.....-A............   [81]
H.aztecanus          ........-......A...G.........--....C.....--.AT....T.....G.TC.A.A......G--.GYA...-A............   [81]
H.zebraneus          ........-.C....A..--.........--....C.....--.AT....T.....G.TC.A.A......G--.G.A...-A.A...--....   [79]
H.virgulatus         ........-.C....A...G......--....A.....--.AT....T.TA...TG.TC.A.A......G--.G.A...-A............   [81]
H.mexicanus          ........-.C....A...G.........GA.....----.T...TTA...TG.TC.A.A......G--.G.A...-A............   [79]
H.borealis           ........T-.C....A...G.........--....A.....--.AT..C.T....TC.TC.A.A......G--.G.A...-A............   [81]
H.coecatus           ........T-.C....A...G.........--....A.....--.AT....T.Y..TG.TC.A.A......G--.G.A...-A............   [81]
H.pyrrithrix         ........-.C....A...G.........--....GA.....----T....T....TG.TC.A.A......G--.G.A...-A............   [79]
H.ballatoris         ........-.C....A...G.........--....A.....--.AT....T....TG.TC.A.A......G--.G.A...-A............   [81]
H.cf.anepsius        ........-.C....A...G.........--....A.....--.AT....T....TG.TC.A.AG.......G--.G.A...-A.........--....   [81]
H.ammophilus         ........-.C....A...G.........--....A.....--.AT....T....TG.TC.A.AG.......G--.G.A...-A.........--....   [81]
```

exon

intron
w/indels

intron
no indels

no changes

1-2 changes

3-4 changes

≥ 5 changes

Sandalodes

H. alachua
H. conjunctus
H. cognatus
H. ustulatus
H. signatus
H. amicus
P. shoshonensis
P. longimanus
H. geronimoi
H. dorotheae
H. huastecanus
H. hallani
H. venatoris
H. carolinensis
H. cockerelli
H. ocala
H. cf. decorus
H. cf. pochtecanus
H. pugillis
H. cambridgei
H. fallax
H. oregonensis
H. icenoglei
H. notialis
H. jucundus
H. c. calcaratus
H. cf. sugillatus
H. texanus
H. altanus
H. tarascanus
H. banksi
H. americanus
H. sansoni
H. tarsalis
H. kawini
H. viridipes
H. c. maddisoni
H. moratus
H. trimaculatus
H. orbus
H. virgulatus
H. borealis
H. mexicanus
H. coecatus
H. cf. anepsius
H. ammophilus
H. ballatoris
H. pyrrithrix
H. velivolus
H. aztecanus
H. forticulus
H. divaricatus
H. zebraneus