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Evidence for Three Major Clades within the Snapping Shrimp Genus *Alpheus* Inferred from Nuclear and Mitochondrial Gene Sequence Data

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The snapping shrimp genus *Alpheus* is among the most diverse of caridean shrimps, and analyses of taxa separated by the Isthmus of Panama have been used to estimate rates of molecular evolution. Although seven morphological groups have been informally suggested, no formal phylogenetic analysis of the genus has been previously attempted. Here we infer the phylogenetic relationships within *Alpheus* using sequence data from two nuclear genes, glucose-6-phosphate isomerase and elongation factor-1α, and from the mitochondrial gene cytochrome oxidase I. Three major clades corresponding to previously noted morphological features were identified. Discrepancies between earlier informal morphological groupings and molecular analyses largely consisted of species whose morphologies were not entirely typical of the group to which they had been assigned. The traditional placements of shrimp with highly sessile lifestyles and consequently simplified morphologies were also not supported by molecular analyses. Phylogenies for *Alpheus* suggest that specialized ecological requirements (e.g., symbiotic associations and estuarine habitats) and modified claw morphologies have evolved independently several times. These new analyses also support the sister species status of transisthmian pairs analyzed previously, although very similar pairs were not always resolved with the more slowly evolving nuclear loci. In addition, six new cryptic species were identified in the course of these studies plus a seventh whose status remains to be determined.

***Key Words: Alpheus; Thunor;* glucose phosphate isomerase; elongation factor-1α; cytochrome oxidase I; phylogeny; chimera; selection.**

The snapping shrimp genus *Alpheus* is among the most diverse and abundant of all caridean shrimp. *Alpheus* can be found in all the world's tropical oceans in a wide variety of habitats, including under stones or in crevices along rocky intertidal shores, in mud associated with estuaries or mangroves, and in symbiotic associations with corals, anenomes, sponges, and fishes. There are approximately 250 described species, but this represents a serious underestimate of the true diversity, because there are numerous cryptic taxa (e.g., Knowlton and Keller, 1985; McClure and Greenbaum, 1994; Bruce, 1999), most of which are as yet undescribed (e.g., Knowlton and Weigt, 1998; see below). Such a genus would benefit from phylogenetic analysis. However, existing taxonomic treatments simply divide *Alpheus* into five or seven informal, broadly distributed species groups that date back to the work of Coutière (1905). These groups are poorly defined, with many morphological exceptions imbedded within them, and have no true systematic status (Kim and Abele, 1988).

Past molecular studies have largely focused on analyses of the mitochondrial cytochrome oxidase (COI) gene in taxa separated by the Isthmus of Panama (Knowlton *et al.*, 1993; Knowlton and Weigt, 1998). These studies have played an important role in the estimation of rates of molecular evolution, but phylogenetic analyses of nontransisthmian pairs were not attempted. To better define phylogenetic relationships at all taxonomic levels in the snapping shrimp genus *Alpheus,* and in particular to confirm the validity of previously suggested morphological groupings, we examined sequence variation at portions of two nuclear genes (elongation factor-1α, EF-1α, and glucose-6-phosphate isomerase, GPI) and at COI. COI sequence variation has proven useful in the past for the identification of transisthmian sister taxa, but is less useful for the resolution of deeper divergences. Therefore we included the two nuclear genes in the hope of increasing the level of resolution of these nodes.

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MATERIALS AND METHODS

Collection of Samples

Most *Alpheus* are free living and were collected from the rocky intertidal shoreline (in crevices or under stones), from within coral rubble close to the low tide level, or from mud flats in or near mangroves. Two groups of obligate symbionts were collected from their hosts (*A. lottini* from living *Pocillopora* or *Stylophora* corals and *A. cylindricus* from sponges). Most samples came from the Pacific and Caribbean coasts of Panama. Smaller collections came from Brazil, the Cape Verde Islands, and the Chagos Archipelago (see Table 1 and figure legends); for the latter two locations we have only COI sequences because frozen samples were not available for RNA extraction. Two species of shrimp currently belonging to the genus *Thunor* were also included to clarify their status, since in the past they have been described as *Alpheus* (Kim and Abele, 1988), and earlier genetic studies suggest that they should be included in *Alpheus* (Knowlton and Weigt, 1998). Table 1 and Fig. 1 summarize the morphological and ecological characteristics of *Alpheus* (and "*Thunor*") examined in this study. For outgroups, we analyzed shrimp belonging to two other genera in the family Alpheidae, *Automate gardineri* and *Alpheopsis trigonus*, collected from the Perlas Islands (Pacific coast of Panama) and San Blas Islands (Caribbean coast of Panama), respectively.

Shrimp were frozen in liquid nitrogen or preserved (for DNA extraction only) either in 95% ethanol or a salt-saturated dimethyl sulfoxide (DMSO) solution (Seutin *et al.*, 1991). Males or nonovigerous females were used in nuclear gene studies to ensure the presence of a single genotype. When inadequate numbers of males and naturally nonovigerous females were available, all eggs were removed from ovigerous females prior to freezing. Additional voucher specimens were preserved in 5% formalin/sea water.

Nuclear and mtDNA Data

Sequences for both nuclear genes examined were obtained from amplification from cDNA for the 62 animals included in the nuclear data sets. Our data indicate that there are multiple copies of COI in *Alpheus* (probably pseudogenes; Williams and Knowlton, 2001); therefore, wherever possible, COI was also amplified from cDNA from specimens included in the nuclear data set, although occasionally from genomic DNA (gDNA) obtained by extraction of the organic phases discarded after RNA extraction. Sequences for COI data from gDNA were also obtained for 50 additional animals. These COI sequences from gDNA were produced by two of the authors (S.W. and L.W.) independently, in different laboratories. Seven previously published COI sequences were also included (GenBank Accession numbers in Fig. 3). COI amplification products were sequenced directly after enzyme cleanup (described below), but nuclear genes were first cloned. Primer sequences are listed in Table 2.

GPI sequence examined is homologous to sequence spanning exons four and five in *Drosophila simulans* (GenBank Accession No. L27549). GPI is a dimeric enzyme (E.C. 5.3.1.9), and each identical subunit folds into two unequal domains, the larger of which evolves more rapidly than the smaller (Fothergill-Gilmore and Michels, 1993). Sequence variation in this study comes from the more rapidly evolving large domain. Sequence data for EF-1α is homologous with the third exon for published sequence for *Artemia salina* (GenBank Accession No. X03705-8). COI sequence is from the center toward the 3′ end of the gene.

RNA and DNA Extraction

Total genomic RNA and DNA were sequentially extracted in a multistep guanidinium thiocyanate/acid phenol:chloroform extraction (Totally RNA extraction kit, Ambion) from whole shrimp (chelae excluded for large animals) as per manufacturer's instructions. Total genomic DNA was also obtained from additional animals (not included in the nuclear data sets) with a PureGene DNA isolation kit, following the manufacturer's protocols, except that samples were digested overnight and sometimes for several days at 65°C with 3–6 μL of Proteinase K (20 mg/ml).

cDNA Synthesis and RT-PCR

First-strand synthesis of cDNA was performed with Superscript II RNase H^- Reverse Transcriptase (Gibco BRL) and a T_{18} primer following manufacturer's instructions, except for the addition of 20 units of recombinant RNasin ribonuclease inhibitor (Promega). An aliquot (usually 2 μL) of cDNA was used in a polymerase chain reaction (PCR) to amplify 445 bp of EF-1α, up to 505 bp of GPI, and up to 677 bp of COI. Reactions were performed in 50-μL volumes containing 0.1 μM forward and reverse primer for each gene, 200 μM each dNTP, 1.5 mM magnesium chloride, 2.5 units of Ampli*Taq* DNA polymerase, and 5 μL of Ampli*Taq* buffer (10×). Thermal cycle parameters were 3 min at 95°C, 5 cycles of 1 min at 94°C, 1 min at 48°C, 80 s at 72°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, 80 s at 72°C, with a 10-min final extension at 72°C.

Cloning

PCR amplicons of nuclear genes were gel-purified prior to being cloned on a 1.2% (w/v) low-melt agarose gel (40 mM Tris–acetate, pH 8, 100 μM EDTA), by excision and treating of the band with Gelase (Epicentre). The liquefied gel sample was further purified with columns from a QIAquick PCR purification kit (Qiagen). Purified products were cloned into JM109 cells with the pGEM-T Easy Vector System II (Promega) following manufacturer's instructions. Individual bac-

TABLE 1

Genetically Distinct Taxa of *Alpheus* Reviewed in This Study, Including Species Previously Placed in the Genus *Thunor*

				Spines			Claw notches			
					Presence		Position			
Taxa	Location	Habitat	Clade	Color of Urop.	Ocul.	Claw	Super.	Infer.	Face	Shape
Alpheus Group 1										
A. peasei	Carib.	Rubble	II	Clear	Yes	Yes	Yes	Yes	No	Twisted
A. amblyonyx	Carib.	Rubble	II	Clear	Yes	Yes	Yes	Yes	No	Twisted
A. macrocheles	CV	Rubble	II	Clear	Yes	Yes	Yes	Yes	No	Twisted
A. dentipes	CV	Rubble	II	Clear	Yes	Yes	Yes	Yes	No	Twisted
Alpheus Group 2										
A. lottini s.s.	E. Pac./IWP	Coral	III	Pigm.	Yes	No	No	No	No	—
A. lottini B	IWP	Coral	III	Pigm.	Yes	No	No	No	No	—
A. websteri	Carib.	Crevice	III	Pigm.	Yes	No	No	No	No	—
A. websteri	E. Pac.	Crevice	III	Pigm.	Yes	No	No	No	No	—
A. thomasi	Carib.	Rubble	III	Pigm.	Yes	No	No	No	No	—
A. malleator	E. Pac.	Crevice	III	Pigm.	Yes	No	No	No	No	—
A. malleator	Carib.	Crevice	III	Pigm.	Yes	No	No	No	No	—
A. normanni	E. Pac.	Sand/rocks	I	Clear	No	No	Yes	No	No	Simple hook
A. normanni A	Carib.	Rubble	I	Clear	No	No	Yes	No	No	Simple hook
A. normanni B	Brazil	Rubble	I	Clear	No	No	Yes	No	No	Simple hook
A. normanni C	Carib.	Rubble	I	Clear	No	No	Yes	No	No	Simple hook
A. formosus A	Carib./Brazil	Rubble	III	Pigm.	Yes	No	No	No	No	—
A. formosus B	Carib.	Rubble	III	Pigm.	Yes	No	No	No	No	—
A. panamensis	E. Pac.	Sand/rocks	III	Pigm.	Yes	No	No	No	No	—
A. sulcatus	E. Pac.	Sand/rocks	??	Clear	No	No	No	No	Yes	Horizontal groove
A. sulcatus	CV	Sand/rocks	??	Clear	No	No	No	No	Yes	Horizontal groove
Alpheus Group 3										
A. cristulifrons	E. Pac.	Rubble/crevice	I	Clear	No	No	No	No	No	—
A. cristulifrons	Carib.	Rubble	I	Clear	No	No	No	No	No	—
Alpheus Group 4										
A. cylindricus	E. Pac.	Sponge	II	Clear	No	Yes	No	No	No	—
A. cylindricus	Carib.	Sponge	II	Clear	No	Yes	No	No	No	—
Alpheus Group 5										
A. paracrinitus "no spot"	E. Pac.	Sand/rocks	I	Clear	No	No	No	No	No	—
A. paracrinitus "no spot" A	Carib.	Rubble	I	Clear	No	No	No	No	No	—
A. paracrinitus "no spot" B	Carib./CV	Rubble	I	Clear	No	No	No	No	No	—
A. rostratus	E. Pac.	Crevice	I	Clear	No	No	No	No	No	—
A. paracrinitus "spot"	Carib.	Rubble	I	Clear	No	No	No	No	No	—
A. paracrinitus "spot"	CV	Rubble	I	Clear	No	No	No	No	No	—
Alpheus Group 6										
A. floridanus A	E. Pac.	Mud	I	Clear	No	No	No	No	No	—
A. floridanus A	Carib.	Mangrove	I	Clear	No	No	No	No	No	—
A. floridanus B	E. Pac.	Mud/rocks	I	Clear	No	No	No	No	No	—
A. floridanus B	Carib.	Mangrove	I	Clear	No	No	No	No	No	—
Alpheus Group 7										
A. bouvieri	E. Pac.	Crevice	I	Clear	No	No	Yes	Yes	No	U-shaped
A. bouvieri	Carib./Brazil	Crevice	I	Clear	No	No	Yes	Yes	No	U-shaped
A. hebes	E. Pac.	Sand/rocks	I	Clear	No	No	Yes	Yes	No	U-shaped
A. hebes	CV	Sand/rocks	I	Clear	No	No	Yes	Yes	No	U-shaped
A. umbo	E. Pac.	Crevice	I	Clear	No	No	Yes	Yes	No	Backward extending
A. schmitti	Carib.	Crevice	I	Clear	No	No	Yes	Yes	No	Backward extending
A. canalis "blue"	E. Pac.	Sand/rocks	I	Clear	No	No	Yes	Yes	No	Backward extending
A. canalis "orange"	E. Pac.	Sand/rocks	I	Clear	No	No	Yes	Yes	No	Backward extending
A. nuttingi	Carib./Brazil	Mud/rocks	I	Clear	No	No	Yes	Yes	No	Backward extending
A. tenuis	E. Pac.	Sand/rocks	I	Clear	No	No	Yes	Yes	No	Backward extending
A. viridari	Carib.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
A. colombiensis	E. Pac.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
A. estuarensis	Carib.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
A. antepaenultimus A	E. Pac.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
A. antepaenultimus B	E. Pac.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
A. chacei	Carib.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
A. latus	E. Pac.	Mangrove	I	Clear	No	No	Yes	Yes	No	Backward extending
"Thunor"										
A. saxidomus	E. Pac.	Crevice	III	Pigm.	Yes	No	No	No	No	—
A. simus	Carib.	Crevice	III	Pigm.	Yes	No	No	No	No	—

Note. Species group numbers refer to informal morphological classifications summarized by Kim and Abele (1988) as follows: Group 1, *macrocheles* or *megacheles* group; Group 2, *sulcatus* or *machrochirus* group; Group 3, *obesomanus* group; Group 4, *crinitus* group; Group 5, *diadema* or *insignis* group; Group 6, *brevirostris* group; and Group 7, *edwardsii* group. Locality abbreviations: Carib., Caribbean coast of Panama; E. Pac., Pacific coast of Panama; CV, Cape Verde Islands; IWP, Indo-West Pacific. Habitats (typical): rubble, found by breaking up dead coral or coralline algae; coral, from living coral; crevice, from confined space within basalt or encrusting coral (east Pacific) or within dead or encrusting coral (Caribbean); mud/rocks, under rocks sitting on mud or sandy mud; sand/rocks, under rocks on sand, muddy sand, or under rocks on cobble beach; sponge, from canals of living sponges; mangrove, from mud near mangroves; mud, dredged from muddy bottom. Clade refers to genetically distinct clades identified in this study (Figs. 3–6). Morphological abbreviations (see Fig. 1 for examples): Urop., uropod spines; Ocul., ocular spines; Claw, spines flanking dactylar articulation of major chela; Super., superior margin of major chela; Infer., inferior margin of major chela; Face, inner or outer lateral face of major chela.

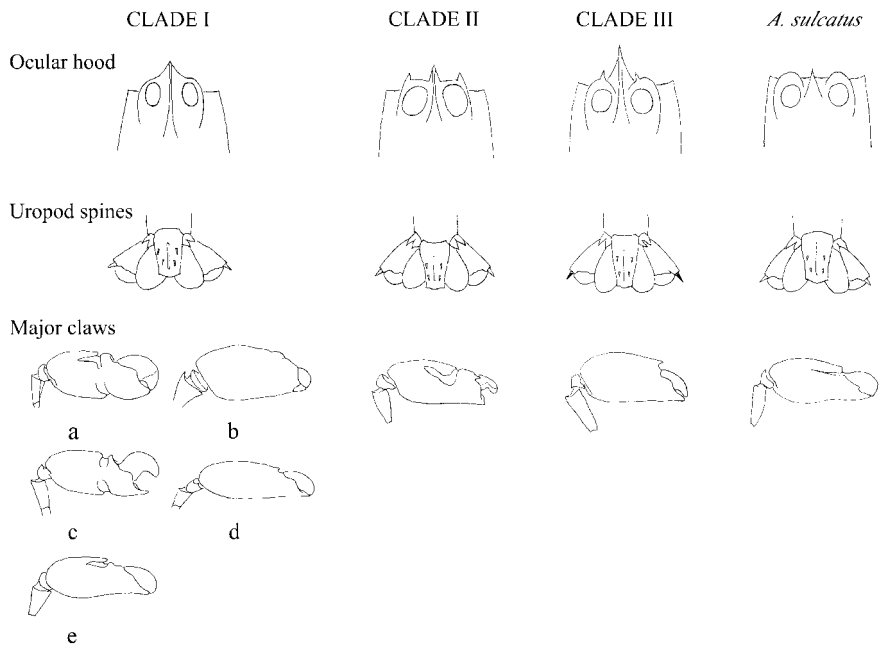

FIG. 1. Major morphological features used to distinguish species that occur in three clades identified in this study and a fourth species (*Alpheus sulcatus*) which does not belong to any of these clades. The main features are (1) presence or absence of spines on occular hoods (carapace extensions that cover eyes), (2) colored (or not) uropod spines, and (3) shape of the major chela. Five types of chela corresponding to (a) the mangrove group and closely related species, (b) the cristulifrons lineage, (c) the bouvieri/hebes lineage, (d) the floridanus and paracrinitus/rostratus lineages, and (e) the normanni lineage (see Fig. 6 for details) occur in Clade I.

terial colonies were picked from the bacterial plate with wide-bore pipette tips and resuspended by being vortexed in 10 μL of water. The presence of an insert of the appropriate size was confirmed by amplification of the insert with 2 μL of the bacteria/water mix and M13 forward and reverse primers in a 25-μL reaction (same concentrations as for RT-PCR) and gel electrophoresis. Cycle parameters were 3 min at 95°C and 30 cycles of 1 min at 94°C, 1 min at 56°C, 80 s at 72°C. Those PCRs with the correctly sized inserts were subjected to an enzyme cleanup, which required incubation of each PCR with eight units of exonuclease I and 1.5 units of

TABLE 2

List of Forward (F) and Reverse (R) Primers Used to Amplify COI, GPI, and EF-1α

Name of primer	Sequence 5′ to 3′	Source
COI primers		
COIF (F)	CCA GCT GGA GGA GGA GAY CC	Kessing *et al.* (1989)
C09 (F)	TTC GGT CAY CCA GAA GTM TAT	Baldwin *et al.* (1998)
C010 (R)	TAA GCG TCT GGG TAG TCT GAR TAK CG	Baldwin *et al.* (1998)
H7188 (R)	CAT TTA GGC CTA AGA AGT GTT G	Knowlton *et al.* (1993)
H7083 (R)	AAT ARG GGG AAT CAG TGG GCA AT	Knowlton *et al.* (1993)
GPI primers		
DS1067 (F)	CGG CCT ACT TCC AGC AGG G	Alignment between GenBank gDNA sequences for copepod[a] and *Drosophila*[b]
DS1574 (R)	AGC TCA ACA CCC CAC TGA TC	Alignment between GenBank gDNA sequences for copepod[a] and *Drosophila*[b]
DS1097 (F)	AAT CTA ATG GAA AGT AYG TAA C	Alignment between *Alpheus* sequences
DS1523 (R)	TGG GTG AAA ATC TTG TGT TC	Alignment between *Alpheus* sequences
EF-1α primers		
EF419 (F)	ACA ACA TGC TGG AGA AGT CAG A	Alignment between *Alpheus* sequence and GenBank cDNA sequences for crab[c] and isopod[d]
EF863 (R)	AGC ACG ATA ACC TGA GCA GTG A	Alignment between *Alpheus* sequence and GenBank cDNA sequences for crab[c] and isopod[d]

Note. GenBank Accession Nos.: [a] U21239; [b] L27549; [c] U90050; [d] U90046.

shrimp alkaline phosphatase for 2 h at 37°C followed by 15 min at 80°C to inactivate the enzymes.

COI PCR from gDNA

Diluted total gDNA (15–80 ng) was used in a PCR to amplify a portion of COI. Reactions were performed as described above for RT-PCR (volume reduced to 25 μL, same thermal cycling profile) or as described in Williams *et al.* (1999). Reactions of the latter variety were performed in 50-μL volumes containing 0.4 μM each primer, 200 μM each dNTP, 1.5 mM magnesium chloride, 1.5 units of Ampli*Taq* DNA polymerase, and 5 μL of Ampli*Taq* buffer (10×). Thermal cycling was performed as follows: initial denaturation for 2 min at 95°C, followed by six cycles of 15 s at 95°C, 15 s at 45°C, 1 min at 72°C, then 30 cycles of 15 s at 95°C, 15 s at 48°C, and 1 min at 72°C with a final extension of 3 min at 72°C. PCR products were sequenced directly after enzyme cleanup (incubation with exonuclease I and shrimp alkaline phosphatase as described above).

Sequencing

Automated sequencing was performed directly on purified PCR products with a dRhodamine Kit (Perkin–Elmer). Protocols for cycle sequencing and consequent purification followed manufacturer's instructions, and the products of sequencing reactions were run on a 377 Applied Biosystems automated sequencer. Between 8 and 20 sequences were generated per individual for each nuclear gene by the sequencing of a single strand from the equivalent number of clones by use of either forward or reverse PCR primers (half with one primer, half with the other). For some species for which the GPI sequence was initially obtained by the cloning of PCR product amplified with primers DS1097 and DS1523, an additional 78 bp was later obtained by the direct sequencing of both strands of a larger PCR product (obtained with DS1067 and DS1574). COI sequences were verified by comparisons of forward and reverse sequences. All sequences have been deposited in GenBank (Accession Nos. for COI: AF308979–AF309001, AF309872–AF309946; for GPI: AF310727–AF310782; for EF-1α first locus: AF310783–AF310836; for EF-1α second locus: AF310837–AF310843).

Phylogenetic Analysis

We constructed trees from consensus nucleotide sequences with a variety of methods using the computer program PAUP* 4.0b3a (Swofford, 1999). Maximum-likelihood (ML) analysis employed a heuristic search, with the tree bisection–reconnection (TBR) branch-swapping method and the start tree derived by neighbor-joining (NJ) with Jukes and Cantor distance data. The most appropriate substitution model for ML was determined with ModelTest 3.0 (Posada and Crandall, 1998). This program runs in conjunction with PAUP* and compares different nested models of DNA substitution in a hierarchical framework and calculates the most appropriate model based on the log-likelihood ratio test statistic (LRT). Codon site-specific rate variation was also tested manually.

Trees were also generated by neighbor-joining with log determinant (LogDet) distances and by weighted maximum-parsimony (MP). MP trees were obtained by stepwise addition of taxa (addition sequence: closest) and the TBR branch-swapping algorithm. Transversions were weighted more heavily than transitions, with the weighting estimated via ML independently for each data set. MP analysis of COI data was also undertaken, excluding third positions. In the combined data set, transversions were weighted twice as heavily as transitions and nuclear genes were arbitarily weighted twice as heavily as COI data to allow for differences in the rate of gene evolution. Trees were constructed independently by all three methods for each gene and with combined sequence data in a total-evidence approach. Bootstrap estimates were also obtained with both NJ and MP with fast stepwise addition (each 1000 replicates).

Saturation Analysis

Mutational saturation of sequences was examined by the plotting of the inferred number of differences against the observed number of differences between each pair of *Alpheus* sequences. Estimated numbers of differences were derived by ML methods (with substitution model used to obtain the tree). Each gene was tested separately.

RESULTS

Features of Sequences

Sequencing of 8 to 20 clones for each individual allowed us to identify alleles accurately. The presence of singletons in cloned nuclear gene sequences (variation unique to a single clone, very rarely to two clones) was noted and attributed to *Taq* polymerase errors (24% of cloned sequences in EF-1α and 28% of cloned sequences in GPI showed evidence of some *Taq* error including base substitutions and/or indels of 1–5 bp, approximate rate of error 7.5%). Our estimates of *Taq* error are high, probably because they were based on clones resulting from multiple cycles of amplification, commencing with RT-PCR, which uses reverse transcriptase, which has a higher rate of replication error than DNA polymerases.

Occasional chimeras were observed, where (usually) three sequences occurred, with one sequence matching the first half of one allele and the last half of the other (see Bradley and Hillis (1997) for another example). We were able to demonstrate experimentally that such sequences were in reality PCR or cloning artifacts. All

three sequences could be generated by reamplification and cloning of a mixture of two PCRs, each resulting from a clone with a different allele. Chimeras were more common in EF-1α than in GPI sequences (eight animals for EF-1α and two for GPI), perhaps reflecting different degrees of difficulty experienced by the *Taq* polymerase enzyme in passing through particular regions of the sequence.

As expected from other studies (France *et al.*, 1999; Duda and Palumbi, 1999), sequence data from some shrimp suggested the presence of a second locus in EF-1α. These sequences, which were neither singletons nor chimeras, were obtained for seven species (including the outgroup species in the genus *Automate*). When included in phylogenetic analyses, these sequences always clustered together, and they were differentiated from other sequences from the same individual by 14–25% (Kimura two-parameter). These sequences were usually obtained from a single clone only, but in *A. lottini* all 16 sequenced clones were from the second locus. Sequences used in the combined data analysis were from one (phylogenetically determined) locus, and the seven additional sequences from the apparent second locus (including that obtained for *A. lottini*) were excluded.

We found 539 variable sites from a total of 1431 bp (564 bp of COI, 466 bp of GPI, and 401 bp of EF-1α) of aligned *Alpheus* nucleotide sequences in the combined data set. Comparison of GPI, EF-1α, and COI revealed that, as expected, COI shows much higher divergences among species than either of the nuclear genes. There were few amino acid replacements in either COI or EF-1α, and pairs of shrimp exhibiting low divergences in COI also exhibited low divergences in EF-1α. All third position sites in COI were polymorphic, but there were only 24 variable amino acid residues among all *Alpheus* sequences examined, half of which showed the same three amino acid substitutions (met-ile, val-ile, phe-leu). In EF-1α there were very few polymorphic amino acid residues (8 within locus 1, 10 within locus 2, and a total of 18 between loci of which 7 are fixed). In GPI, however, a large proportion of nucleotide substitutions resulted in amino acid replacements (47 polymorphic amino acids).

Every third position in the COI sequences was variable, and sequences start to show some evidence of saturation above a value of about 50 observed differences, equivalent to an inferred difference (calculated by ML) of 90, reaching a plateau at about 105 observed differences (Fig. 2a). This means that the data are showing some evidence of saturation below the level of differentiation of most transisthmian sister species and high levels of saturation between the more differentiated geminates and in all other comparisons. On the other hand, although both GPI and EF-1α deviate from the expected line of no saturation, they show less evidence of saturation than COI (Figs. 2b and 2c).

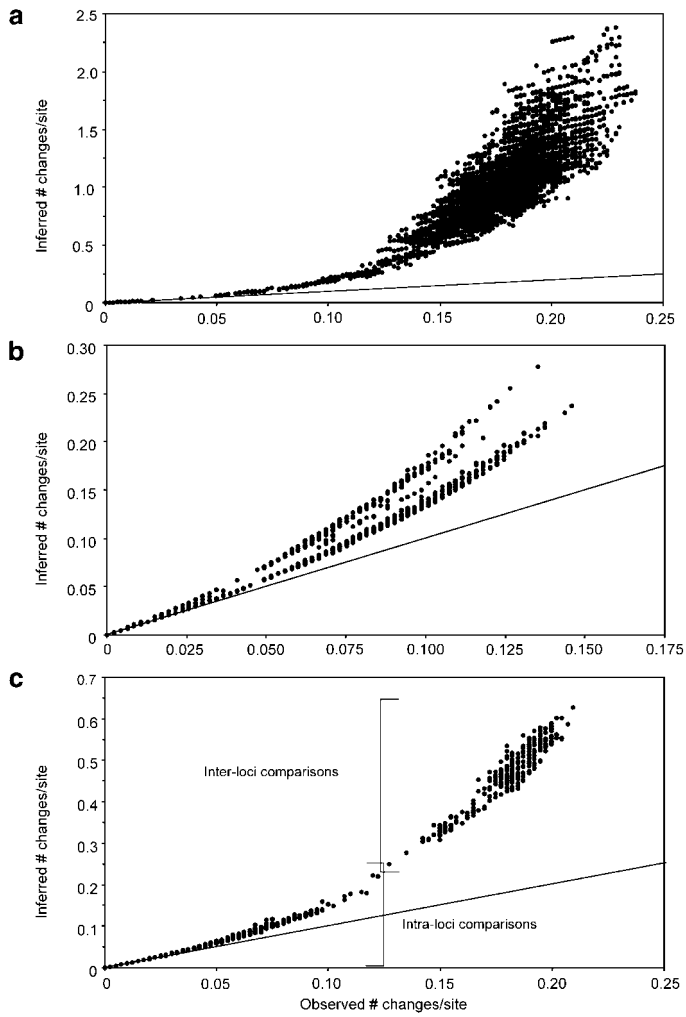

FIG. 2. Saturation curves for *Alpheus* (a) COI, (b) GPI, and (c) EF-1α data. On the X axis is the observed number of differences per site between pairs of *Alpheus* sequences and on the Y axis is the number of inferred (by ML) substitutions. The lines indicated represent the relationship expected if there was no saturation.

Saturation in EF-1α is most apparent when sequences from different loci are compared (Fig. 2c). The multiple curves observed in the GPI graph (Fig. 2b) represent pairwise comparisons between sequences of different lengths (arising from the use of different primer pairs, see Materials and Methods). All pairwise analyses of all samples fall in the lower line, except for those analyses that include one of seven shorter sequences. The middle line is all pairwise analyses including one "medium"-length sequence.

Phylogenetic Analysis of Larger Groups within Alpheus

Of the 539 variable sites, 461 were phylogenetically informative [227 in COI, 155 in GPI, and 120 in EF-1α (individual data sets)]. Phylogenetic analyses of COI, GPI, EF-1α, and the combined data set all produced

similar results; therefore, we present only the ML trees (Figs. 3–6). Topologies observed with all methods of analysis are listed in Table 3.

The model chosen by ModelTest maximum-likelihood analysis of the COI data set was the Hasegawa–Kishino–Yano (1985) model (HKY) with rate variation and invariable sites. This model allows for inequality of base frequencies and transition/transversion rates. Rates of substitution were also considered to vary among sites following a gamma distribution (four categories). The transition/transversion ratio (7.83), gamma shape parameter ($\alpha = 0.60$), proportion of constant sites (I = 0.57), and base frequencies (A: 0.39, C: 0.26, G: 0.10, T: 0.26) for this and other data sets were estimated via ML.

GPI was also analyzed by the HKY model with rate variation. This model estimates base frequencies and allows for inequality of frequencies (A: 0.32, C: 0.21, G: 0.20, T: 0.27) and transition/transversion rates (ratio = 2.15) and variation in rate among sites ($\alpha = 0.37$).

The model chosen for EF-1α (both loci–outgroups excluded) was that of Tamura and Nei (1993) (TrN) with equal base frequencies, rate heterogeneity ($\alpha = 0.59$), and invariable sites (I = 0.46). This model allows different rates of transition for purines (3.04) and pyrimidines (6.58).

The combined data set included only samples that had sequence data for all three genes. The model chosen for the combined data (outgroups excluded) was the TrN model with rate heterogeneity ($\alpha = 0.39$), invariable sites (I = 0.51), and unequal base frequencies (A: 0.33, C: 0.27, G: 0.14, T: 0.26). This model estimates rates of transition, allowing different rates for purines (8.74) and pyrimidines (10.06).

Three major clades were consistently identified by all analyses except MP analysis of COI data, in which only Clade III was resolved. However, the topology of major lineages within Clade I, the placement of Clade II with respect to Clades I and III, and the placement of members of the *Alpheus sulcatus* lineage (indicated by an asterisk in Figs. 3–6) varied somewhat depending on the gene examined and the method of analysis used (Table 3).

A. sulcatus is the only lineage for which there is no strong evidence as to which (if any) of the three clades it belongs. Most analyses suggest that it is an outgroup to all the other *Alpheus* species analyzed (Table 3). In EF-1α, *A. sulcatus* is placed as an outgroup to either Clade I or Clade II or Clades II and III (Fig. 4). The placement of *A. sulcatus* does not receive strong bootstrap support in any location.

Clade I has the same species composition in all analyses in which it is monophyletic. In all analyses, a group of shrimp associated with mangroves (the *A. estuarensis, A. columbiensis, A. chacei,* and *A. antepaenultimus* lineages) are monophyletic within Clade I. For both nuclear loci, the closest relatives of the mangrove group were the *A. schmitti/umbo, A. tenuis, A. nuttingi/canalis,* and *A. cristulifrons* lineages, with the latter generally being the most ancestral (well supported only in the combined data set). Clades II and III have the same species composition in all analyses with the exception of *A. sulcatus* (as discussed above).

The relationships among the three clades are not consistent across all genes and analyses. However, all analyses of combined data and GPI and two of three analyses of EF-1α suggest that Clade II is the sister group of Clade III. Moreover, three of three informative amino acid substitutions (one for COI, two for GPI) are consistent with this grouping.

Recognition of Cryptic Species and Transisthmian Sister Species Pairs

Sequence data in some cases revealed evidence for the existence of additional cryptic species not reported in earlier studies (Knowlton *et al.*, 1993; Knowlton and Weigt, 1998). Taxa that are more genetically distinct than transisthmian sister species should definitely be recognized at the species level because studies of the latter have shown them to be reproductively isolated. Nuclear sequence data also support the distinctiveness of cryptic taxa previously identified by color pattern, COI, and allozyme differences (Knowlton *et al.*, 1993).

The discovery of additional cryptic taxa probably stems from the fact that shrimp analyzed for this study came from areas (both within and outside Panama) that had not been previously sampled. New taxa from the eastern Pacific and western Atlantic are affiliated with *A. floridanus* (one additional taxon in both the eastern Pacific and the Caribbean, species pair B), *A. antepaenultimus* (one additional taxon in the eastern Pacific, species B), *A. paracrinitus* "no spot" (one additional taxon in the western Atlantic, species B), and *A. normanni* (one additional taxon in the Caribbean, species C, and one from Brazil, species B). All taxa from the Cape Verde Islands (including taxa supposedly conspecific with eastern Pacific or western Atlantic forms) were also genetically distinct, except for a member of the *A. paracrinitus* "no spot" lineage (which is very similar to the new Caribbean form, species B). The taxonomic status of a genetically distinct form of *A. nuttingi* from Brazil is uncertain since it shows less differentiation from the Panamanian form than that observed between transisthmian geminates.

Generally, these new cryptic species do not change the conclusions of previous studies (Knowlton *et al.*, 1993; Knowlton and Weigt, 1998), because they are more distantly related to previously identified transisthmian sister species than the latter are to each other. For example, the previously identified species of *A. antepaenultimus* (species A) remains the true geminate to *A. chacei,* and a new Caribbean species of *A. paracrinitus* "no spot" is more similar to the Cape Verde specimens than to related transisthmian taxa.

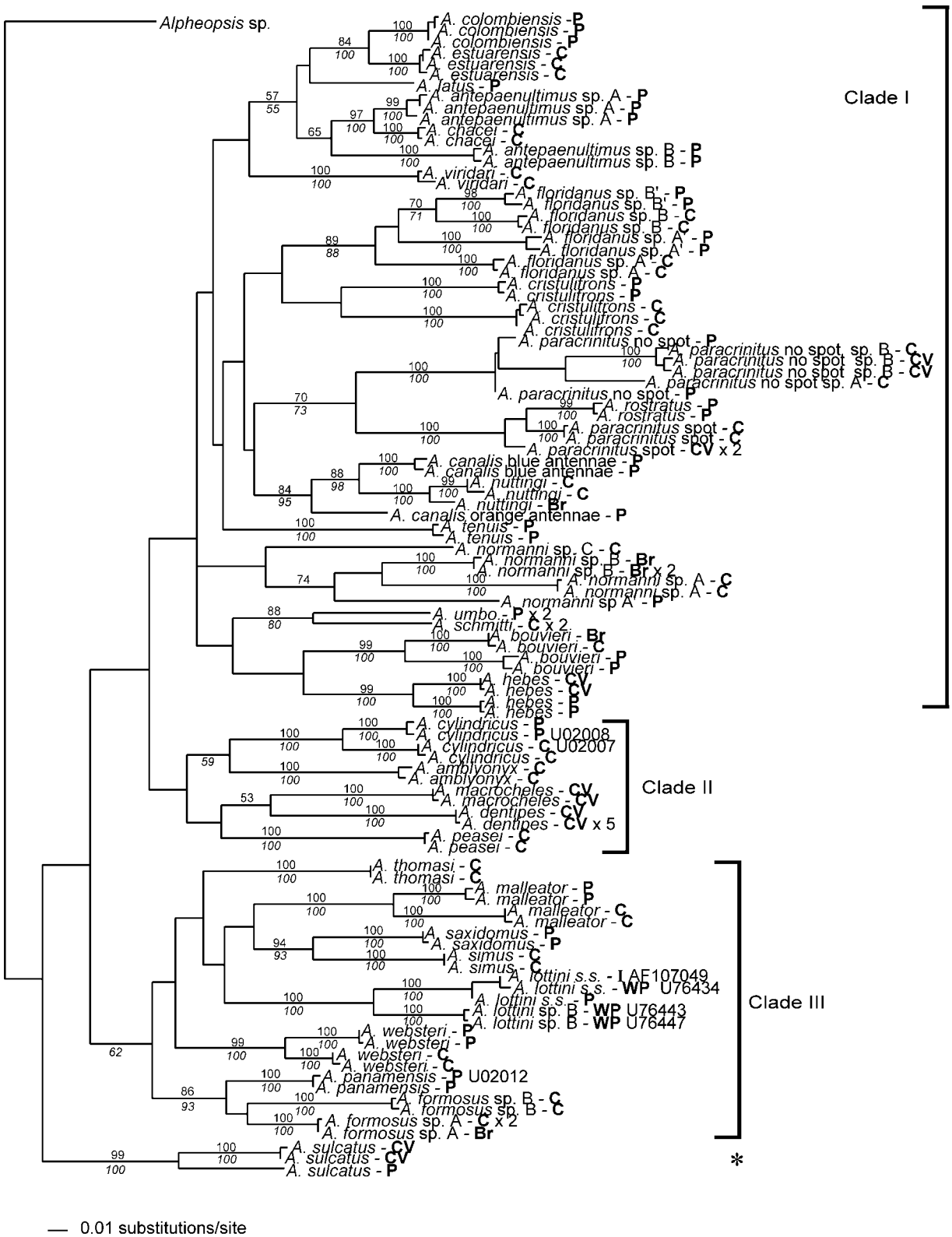

FIG. 3. ML tree (LnL = 10666) for snapping shrimp COI sequences. Numbers above each branch are MP bootstrap values based on fast heuristic approach with transversions weighted three times as heavily as transitions; numbers in italics below each branch are NJ bootstrap support values based on LogDet distances. Only values >50% and for interspecific comparisons are shown (species are here defined as genetically distinct units that are as genetically differentiated as transisthmian taxa). OTU's are identified by species name, additional descriptors, and, after the hyphen, location (abbreviation in boldface) and number of identical sequences found. For previously published sequences the GenBank accession number is given. P, Pacific coast of Panama; C, Caribbean coast of Panama; CV, Cape Verde; Br, Brazil; for *A. lotttini* only: WP, West Pacific; I, Chagos Islands, Indian Ocean. Identical sequences are identified by the listing of (where appropriate) multiple geographic locations and/or numbers which indicate the number of sequences from a given location.

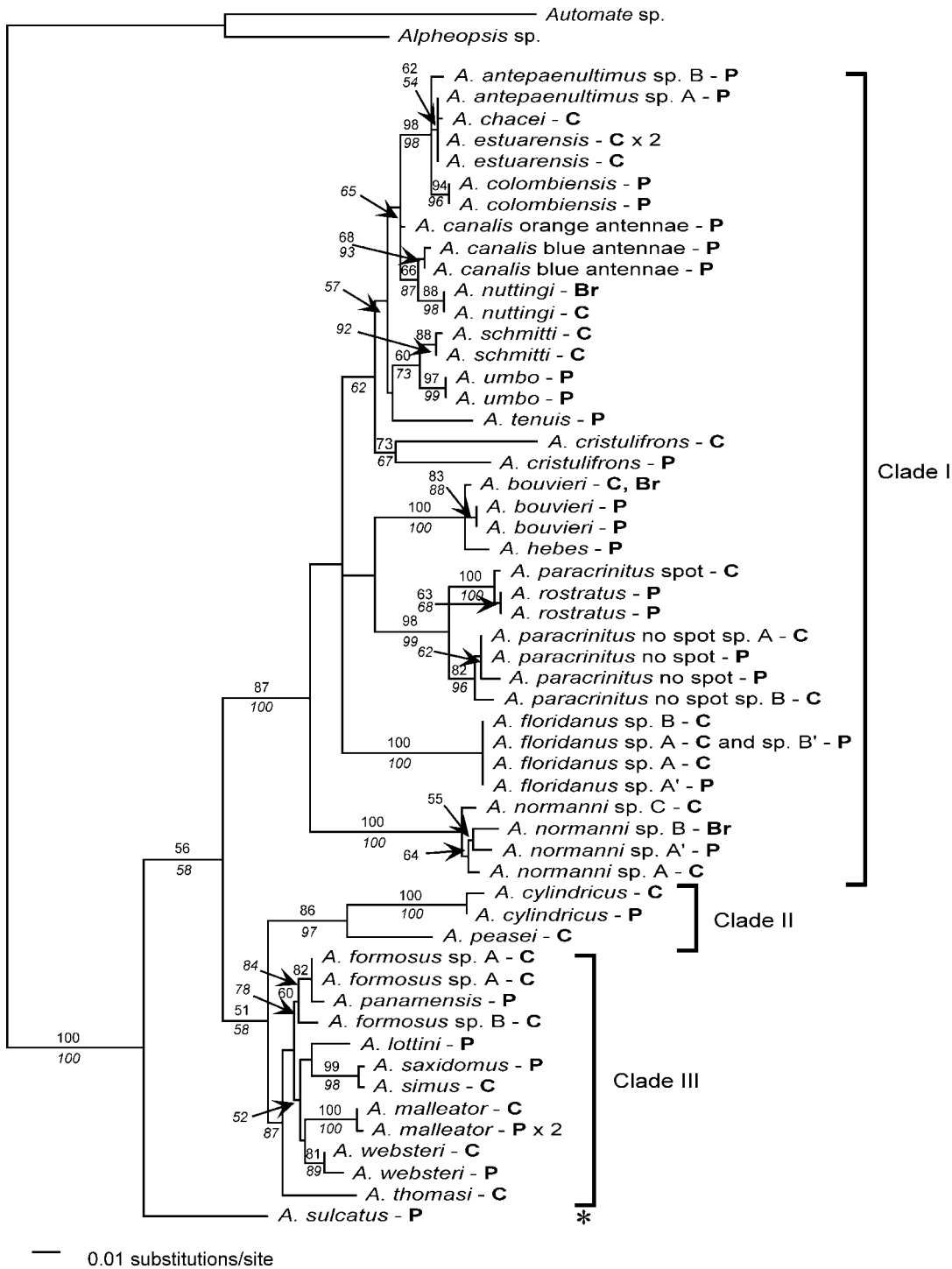

FIG. 4. One of two ML trees obtained (both with LnL = 3013) for snapping shrimp GPI sequences. The other tree differed only in that it reversed the positions of the two sequences for *A. paracrinitus* (no spot, Pacific). Numbers above each branch are MP bootstrap values based on fast heuristic approach with transversions weighted two times as heavily as transitions; numbers in italics below each branch are NJ bootstrap support values based on LogDet distances. Only values >50% are shown. OTU's are identified as in Fig. 3. Identical sequences are indicated by the listing of (where appropriate) names of species and number of individuals.

However, the new second pair of *A. floridanus* (species pair B) represents a new, more closely related geminate pair than the previously identified pair (species pair A). Also, *A. normanni* (species B) from Brazil appears to be the true sister species of the eastern Pacific taxon, and *A. nuttingi* from Brazil is slightly

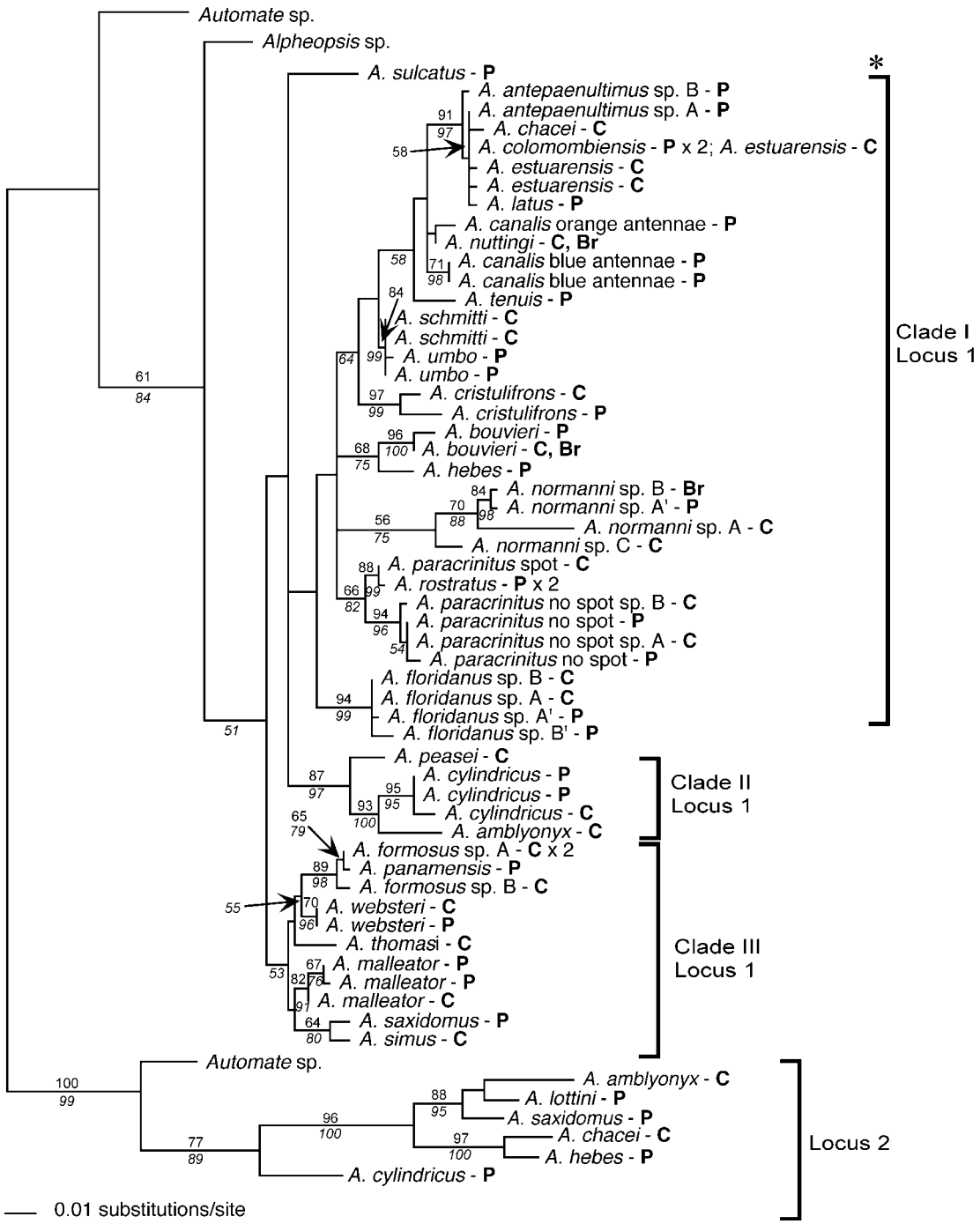

FIG. 5. One of two ML trees obtained (both with LnL = 2727) for snapping shrimp EF-1α sequences. The other tree differed only in that the two *A. estuarensis* sequences group together. Numbers above each branch are MP bootstrap values based on fast heuristic approach with transversions weighted two times as heavily as transitions; numbers in italics below each branch are NJ bootstrap support values based on LogDet distances. Only values >50% are shown. OTU's are identified as in Fig. 3. Identical sequences are indicated by the listing of (where appropriate) names of species and number of individuals.

less divergent from the eastern Pacific *A. canalis* "blue" than is *A. nuttingi* from the Caribbean coast of Panama.

Both the COI (Fig. 3) and the combined data (Fig. 6) ML analyses identify the same transisthmian species pairs, which, with the exceptions noted above, are identical to those previously identified by COI, allozymes, and morphology (Knowlton *et al.*, 1993; Knowlton and Weigt, 1998). In most cases, nuclear genes analyzed on their own gave consistent results, but in several cases,

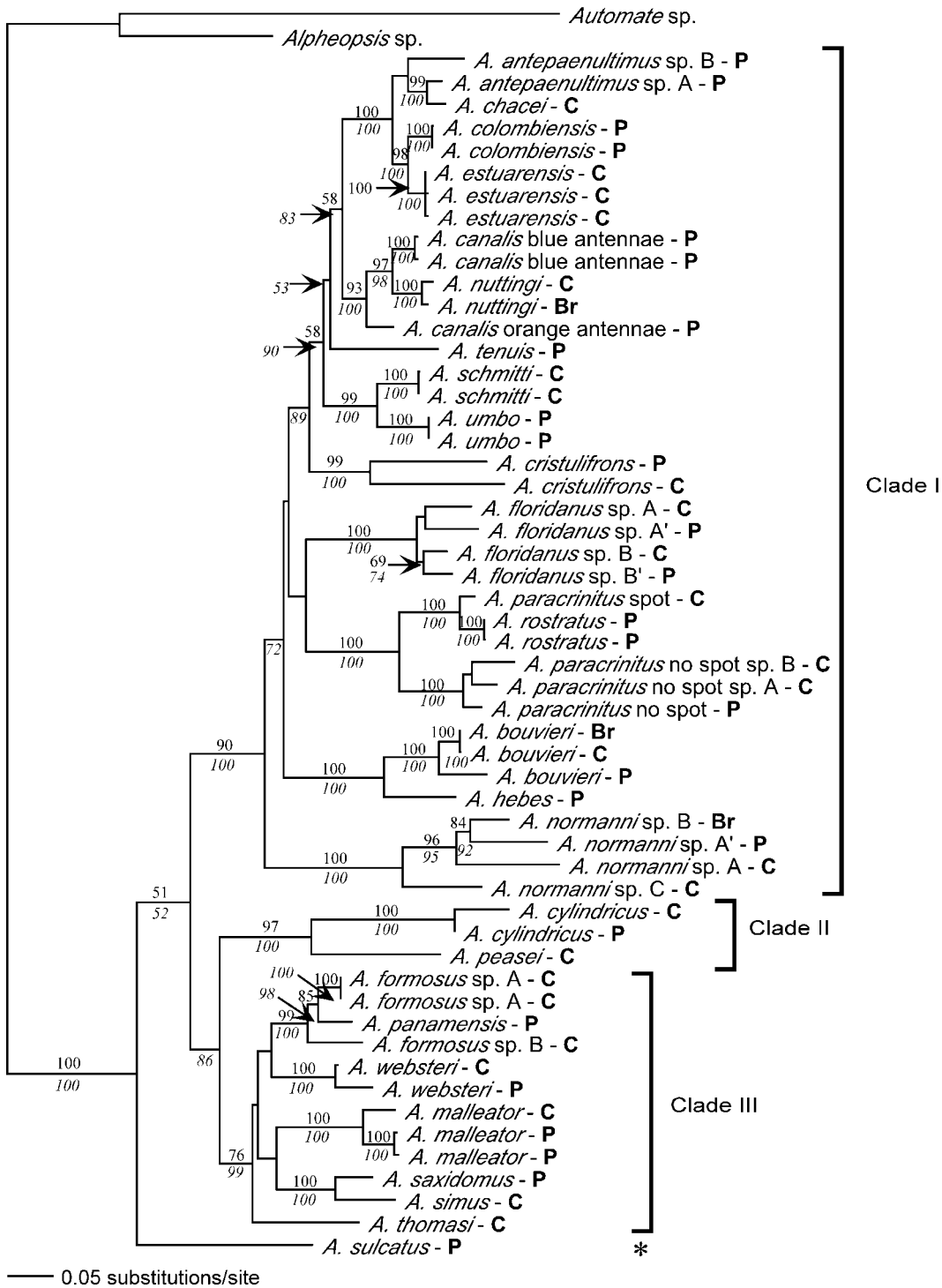

FIG. 6. ML tree (LnL = 14243) for snapping shrimp combined COI, GPI, and EF-1α sequences. Numbers above each branch are MP bootstrap values based on fast heuristic approach and numbers in italics below each branch are NJ bootstrap support values based on LogDet distances. Transversions were weighted twice as heavily as transititions in MP analysis, and nuclear genes were weighted twice as heavily as COI. Only bootstrap values >50% are shown. OTU's are identified as in Fig. 3.

the resolution of the nuclear genes was not sufficient to identify transisthmian sister species (Figs. 4 and 5). For example, the two pairs of mangrove transisthmian taxa (*A. colombiensis*/*A. estuarensis* and *A. antepaenultimus* sp. A/*A. chacei*) and the two pairs within the *A. floridanus* lineage were not well resolved by either

TABLE 3

Summary of Results of Phylogenetic Analyses by Gene and Type of Analysis

Gene and analysis	Relationship among clades	Placement of *sulcatus* lineage
COI ML	(I+II) III	Outgroup to other *Alpheus*
COI NJ	(I+II) III	Within Clade I
COI MP	—	Outgroup to other *Alpheus*
GPI ML	(II+III) I	Outgroup to other *Alpheus*
GPI NJ	(II+III) I	Outgroup to other *Alpheus*
GPI MP	(II+III) I	Outgroup to other *Alpheus*
EF 1α ML	(I+II) III	Outgroup to Clade I
EF 1α NJ[a]	(II+III) I	Outgroup to Clades II and III
EF 1α MP	(II+III) I	Outgroup to Clade III
Combined ML	(II+III) I	Outgroup to other *Alpheus*
Combined NJ	(II+III) I	Outgroup to other *Alpheus*
Combined MP	(II+III) I	Outgroup to other *Alpheus*

[a] Locus one only.

EF-1α or GPI. In addition, EF-1α linked *A. nuttingi* with *A. canalis* "orange" rather than with *A. canalis* "blue."

DISCUSSION

Utility of Molecular Markers

GPI has been commonly used in protein electrophoretic studies [including transisthmian *Alpheus* (Knowlton *et al.,* 1993)], but this is the first phylogenetic study of animals with nucleotide sequence data. GPI is an essential enzyme in the glycolysis pathway, where it catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate. More recently, it has been found to function in vertebrates as a neurotrophic factor (Chaput *et al.,* 1988; Faik *et al.,* 1988; Gurney, 1988), an autocrine motility factor (Watanabe *et al.,* 1996; Niinaka *et al.,* 1998), and a maturation factor (Xu *et al.,* 1996; Niinaka *et al.,* 1998). GPI sequence was very informative at deeper nodes and surprisingly capable of identifying transisthmian sister species at branch tips. Nevertheless, our analyses suggest that some caution must be used in phylogenetic analyses based on GPI, because some evidence for nonneutral evolution exists at this locus.

Tests in which log-likelihood ratios were compared to examine whether the dN/dS ratios (where dN is the number of amino acid-altering nucleotide substitutions per nonsynonymous site and dS is the number of silent substitutions per synonymous sites) differ among lineages suggest that ratios are indeed different among lineages (S. T. Williams, unpublished). Heterogeneity of dN/dS ratios among lineages may be caused by either positive selection or relaxed selection in some lineages (Yang, 1998). Previous studies have linked variation in GPI allozyme electromorphs with variation in temperature and related factors, including salinity, pH, exposure, pollution, and oxygen availability (reviewed in Riddoch, 1993). GPI genotypes have also been shown to differ in their stability and activity over a range of temperatures in several invertebrates (e.g., Hall, 1985; Zamer and Hoffman, 1989; Ward, 1992) and in some cases these genotypes appear to affect survivorship and mating success (e.g., Ward, 1992).

Elongation factor-1α (EF-1α) has been widely used to infer phylogenies (e.g., Cho *et al.,* 1995; Belshaw and Quicke, 1997; Friedlander *et al.,* 1998). It is one of the most abundant proteins in eukaryotic cells, in which it catalyzes the binding of charged tRNAs to the ribosome during translation. A potential problem with the usefulness of EF-1α as a phylogenetic marker in shrimp was the apparent occurrence of two loci [as reported in several other arthropods (France *et al.,* 1999; Duda and Palumbi, 1999; Lenstra *et al.,* 1986; Hovemann *et al.,* 1988)] and the possibility of pseudogenes [e.g., as in humans (Uetsuki *et al.,* 1989)]. We believe that the sequences found in this study represent two functional loci, since our sequences were amplified from cDNA, suggesting that all sequences are from genes that are expressed. Fortunately, the clear reciprocal monophyly of the two loci in *Alpheus* means that it is possible to differentiate between loci (contrary to France *et al.,* 1999). In contrast to GPI, there were few amino acid substitutions associated with nucleotide differences in EF-1α. EF-1α sequences yielded a ML tree with weaker bootstrap support for some of the deeper nodes than did GPI, which may be the result of insufficient numbers of phylogenetically informative sites. There is, however, enough resolution to obtain a tree with essentially the same topology as those obtained for GPI.

COI sequences contained more phylogenetically informative sites than either nuclear gene (216/564 in an equivalent data set), but saturation limited its utility to shallower divergences (e.g., those provided by transisthmian pairs and other closely related taxa). As with EF-1α, there were few amino acid replacements.

Combining the data sets increased the number of phylogenetically informative sites and resulted in a robust phylogenetic hypothesis for both deep and shallow nodes with a variety of types of analyses. Simultaneous analysis of all data sets provides the severest test of a phylogenetic hypothesis, by attempting to explain the distribution of all characters from all genes in the most parsimonious manner (Kitching *et al.,* 1998, pp. 167–168).

Relationships among Patterns of Morphology, Ecology, and Molecular Phylogeny

Genetic analysis of the three independent loci and preliminary data for a fourth (glutamine synthetase; S. T. Williams, unpublished) consistently revealed three major clades in *Alpheus;* although a formal re-

analysis of morphological characters to compare with molecular phylogenies is beyond the scope of this study, several concordant features can be noted. Most importantly, members of the same clade share certain conspicuous morphological features, and members of the same morphological species group (Kim and Abele, 1988) were usually assigned to the same molecular clade (Table 1). Clade I broadly corresponds to shrimp without ocular spines, which includes all members of four morphological species groups plus one lineage within a fifth species group. Clade II corresponds to shrimp with ocular spines and spines flanking the dactylar articulation of the major chela and comprises all members of two morphological species groups. Clade III comprises shrimp with ocular spines and pigmented uropod spines and includes most members of one species group plus shrimp formerly assigned to the genus *Thunor*. The majority of molecular analyses suggest that Clades II and III are sister groups, and this is supported morphologically by the presence of ocular spines in both groups. Molecular data suggest that the *A. sulcatus* lineage may be an outgroup to the rest of the genus, and several unusual morphological features are also consistent with this hypothesis (e.g., claw notches; see Table 1).

The major discrepancies between earlier morphological groupings and our molecular phylogenies consisted of shrimp that were previously noted as lacking features typical of the group (e.g., placement of *A. normanni* and *A. sulcatus* in species group 2, despite the absence of both ocular spines and pigmented uropod spines; Table 1). Phylogenetic placements of shrimp with very reduced morphologies associated with sessile lifestyles (the *A. cylindricus* and "*Thunor*" lineages) were also clarified. Our molecular analyses suggest that their unusual morphologies (and their lack of ocular spines) represent secondary changes associated with protection within the confines of a host or a rock cavity. Moreover, reexamination of these shrimp reveals that they do share characteristic morphological features of the molecular clades to which they are assigned—dactylar spines in the case of *A. cylindricus* (albeit reduced) and pigmented uropod spines in the case of "*Thunor*."

Clade I exhibits the greatest morphological and genetic diversity; although all these shrimp lack ocular spines, they vary widely in claw morphology and originally were divided among five species groups (Table 1). The ML tree for the combined data set suggests that the *A. floridanus* and *A. paracrinitus/rostratus* lineages are more closely related to each other than other lineages and both lineages have chela which lack notches (chela type "c" in Fig. 1); however, this receives only poor bootstrap support (<50%; Fig. 6). In general, molecular data do not resolve well the relationship among the *A. floridanus, A. normanni, A. bouvieri/hebes,* and *A. paracrinitus/rostratus* lineages. In the case of COI this is probably due to the accumulation of multiple substitutions, which has erased the phylogenetic signal at deeper nodes. However, the same problem with analyses using the two nuclear genes, in the absence of strong evidence of saturation, suggests that these groups may have diverged at approximately the same time. Alternatively, failure to obtain a clear phylogenetic pattern may reflect the fact that there are few species belonging to these groups found in the eastern Pacific and Atlantic; addition of taxa from other regions may prove useful in the defining of relationships. Group 7 is very diverse in this region, but our genetic studies indicate that its composition as conventionally defined (by the presence of superior and inferior claw notches; Table 1) is not monophyletic. Indeed, although tree topology is not strongly supported, notches or grooves on the claw appear to have evolved on three separate occasions (the *A. bouvieri/hebes* lineage, the other members of morphological group 7, and the *A. normanni* lineage). In retrospect, this is not surprising, since the morphologies of these notches differ among them (Table 1, Fig. 1). Further analyses with additional species related to these taxa should help to clarify the patterns of claw morphology relative to phylogeny.

These phylogenetic analyses also lend insight into ecological radiations within this diverse genus. Just among the taxa analyzed (a small portion of the total diversity), symbiosis has evolved twice (the *A. cylindricus* and the *A. lottini* lineages), as has the ability to live in low-salinity habitats (the *A. columbiensis/A. estuarensis/A. chacei/A. antepaenultimus* lineage and the Caribbean members of the *A. floridanus* lineage) and the ability to live in confined cavities (the *A. umbo/schmitti* and the *A. malleator* + *A. simus/A. saxidomus* lineages). This suggests considerable ecological flexibility within the genus, which may contribute to its overall success in tropical and subtropical habitats.

Taxonomic Implications

The existence of three clear lineages within the genus poses the question of whether the genus itself should be split into several genera. Indeed, application of one standard outlined by Avise and Johns (1999) would result in many transisthmian pairs being placed in different genera! Nevertheless, we are reluctant to recommend splitting the genus at this point, because it would result in the loss of the ability to retrieve information about a well-known and reliably recognized group. We do, however, recommend that the three major clades be given subgeneric status and that the traditional morphological groupings be abandoned. A decision about subgeneric status for the *sulcatus* lineage should await additional analyses designed to clarify its position with respect to the three main clades. Identification of groups within clades will also have to await further analyses with additional taxa from the

Indo-West Pacific. This is especially true for the four most poorly resolved lineages (*A. floridanus, A. normanni, A. bouvieri/hebes,* and *A. paracrinitus/rostratus*) for which we have only a few taxa.

Finally, many of the geminate taxa divided by the isthmus of Panama are currently recognized as different species, including the two genetically most similar pairs (*A. antepaenultimus/A. chacei* and *A. colombiensis/A. estuarensis*); however, many other pairs, are not (e.g., *A. normanni*). Previous studies of seven transisthmian pairs showed that they can be separated by morphology and exhibit evidence of reproductive isolation in sympatry (Knowlton *et al.*, 1993). Thus, we suggest here formally that all geminate shrimp merit description as separate species. For similar reasons, any genetic group that shows equal or greater genetic divergence than does transithmian shrimp should also be recognized as separate species. This can be justified because not only were transisthmian species reproductively isolated, but levels of aggression between taxa are positively correlated with genetic distance (based on COI data) (Knowlton *et al.*, 1993). Most new species can be recognized by morphological distinctions and many were found in sympatry with their closest relative (e.g., *A. paracrinitus* no spot spp. A and B both occur in the Caribbean). We also formally suggest that *Thunor saxidomus* and *T. simus* be moved back into the genus *Alpheus*.

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