

# Evolution and Phylogenetic Utility of Alignment Gaps Within Intron Sequences of Three Nuclear Genes in Bumble Bees (*Bombus*)

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To test whether gaps resulting from sequence alignment contain phylogenetic signal concordant with those of base substitutions, we analyzed the occurrence of indel mutations upon a well-resolved, substitution-based tree for three nuclear genes in bumble bees (*Bombus*, Apidae: Bombini). The regions analyzed were exon and intron sequences of long-wavelength rhodopsin (LW Rh), arginine kinase (ArgK), and elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) F2 copy genes. LW Rh intron had only a few uninformative gaps, ArgK intron had relatively long gaps that were easily aligned, and EF-1 $\alpha$  intron had many short gaps, resulting in multiple optimal alignments. The unambiguously aligned gaps within ArgK intron sequences showed no homoplasy upon the substitution-based tree, and phylogenetic signals within ambiguously aligned regions of EF-1 $\alpha$  intron were highly congruent with those of base substitutions. We further analyzed the contribution of gap characters to phylogenetic reconstruction by incorporating them in parsimony analysis. Inclusion of gap characters consistently improved support for nodes recovered by substitutions, and inclusion of ambiguously aligned regions of EF-1 $\alpha$  intron resolved several additional nodes, most of which were apical on the phylogeny. We conclude that gaps are an exceptionally reliable source of phylogenetic information that can be used to corroborate and refine phylogenies hypothesized by base substitutions, at least at lower taxonomic levels. At present, full use of gaps in phylogenetic reconstruction is best achieved in parsimony analysis, pending development of well-justified and generally applicable methods for incorporating indels in explicitly model-based methods.

## Introduction

Phylogenetic analysis of nucleotide and amino acid sequence data often requires alignment of homologous sequences that vary in length. As a result, gaps are introduced to the data matrix, representing putative insertion or deletion events. As the products of particular evolutionary processes (mutations), indels are often considered as a class of phylogenetic characters to be incorporated in phylogenetic analysis (Wheeler 1996, 1999; Lutzoni et al. 2000; Simmons and Ochoterena 2000) or to be used to corroborate results derived from base substitutions (van Dijk et al. 1999; Rokas and Holland 2000). However, in most phylogenetic analyses gaps are ignored as missing data, or regions containing gaps are simply excluded from data sets. One reason for discarding gap characters in phylogenetic analyses is that use of gaps as characters is generally confined to the parsimony method, since well-justified and generally applicable methods for incorporating indels have yet to be developed for methods based on explicit models of sequence evolution, such as standard implementations of maximum likelihood (see e.g., Swoford et al. 1996). Another reason to exclude gaps is that their positions are often difficult to determine, especially when analyzing a broad range of taxa and/or highly diverged sequences. Phylogenetic reconstruction is highly sensitive to different alignment options (e.g., gap-to-substitution costs or alignment algorithms), which can lead to very different phylogenetic hypotheses (Giribet and Wheeler 1999; Sanchis et al. 2001). As a novel approach within the parsimony framework, Wheeler

(1996) developed a direct optimization method for searching most parsimonious trees without prior sequence alignments. Subsequently, Wheeler (1999) and Lutzoni et al. (2000) independently developed similar methods for accommodating ambiguously aligned sequences without violating positional homology.

Despite recent progress in analyzing gap characters, these have not been widely accepted as phylogenetic markers due, in part, to insufficient empirical study of the quality of gaps as characters. Some authors assume that gaps are less homoplastic and therefore more phylogenetically reliable than base substitutions, since gaps generally occur less frequently (Lloyd and Calder 1991; Giribet and Wheeler 1999; van Dijk et al. 1999). However, others emphasize the potential for gaps to be misleading (Bapteste and Philippe 2002). Despite the need for further critical evaluation of gaps as phylogenetic characters, few studies have focused on investigating homoplasy levels of gaps as compared with base substitutions or assessing the contribution of gaps to phylogenetic resolution and nodal support (Graham et al. 2000; Simmons, Ochoterena, and Carr 2001; Bapteste and Philippe 2002). Additional empirical study of relative homoplasy levels among different types of gap characters and of the degree to which these contribute to phylogenetic reconstruction would facilitate the full and appropriate utilization of information potentially available in variable-length sequence data.

In this study, we tested the potential of gaps as phylogenetic characters by analyzing their occurrence upon a well-resolved, substitution-based tree and assessing their contribution to further resolution among 66 species (23 subgenera) of bumble bees (*Bombus*). *Bombus* is a diverse, monophyletic genus comprising nearly 250 species (38 subgenera) (Williams 1998) and is therefore an ideal group for studying patterns of indel evolution among species and subgenera. We analyzed exon and intron

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sequences of three nuclear genes: long-wavelength rhodopsin (LW Rh), arginine kinase (ArgK), and elongation factor-1 $\alpha$  F2 copy (EF-1 $\alpha$ ).

## Materials and Methods

A list of exemplar species studied, collection data for each exemplar, and GenBank accession numbers are given in supplementary online materials. Genomic DNA was extracted from thoracic muscles using the standard phenol-chloroform method. We PCR-amplified a total of  $\sim 2.4$  kb of LW Rh, ArgK, and EF-1 $\alpha$  F2 copy genes using the following forward (F) and reverse (R) primers: LW Rh, (F) 5'-AAT TGC TAT TAY GAR ACN TGG GT-3' and 5'-ATA TGG AGT CCA NGC CAT RAA CCA-3' (Mardulyn and Cameron 1999); ArgK, (F) 5'-GTT GAC CAA GCY GTY TTG GA-3' and (R) 5'-CAT GGA AAT AAT ACG RAG RTG-3' or (F) 5'-GA CAG CAA RTC TCT GCT GAA GAA-3' and (R) 5'-GGT YTT GGC ATC GTT GTG GTA GAT AC-3'; EF-1 $\alpha$ , (F) 5'-GGA CAC AGA GAT TTC ATC AAR AA-3' and (R) 5'-TTG CAA AGC TTC RTG RTG CAT TT-3'. PCR products were directly sequenced using the above primers.

For initial alignment, we used ClustalX version 1.81 (Jeanmougin et al. 1998) with the default parameter settings. The alignments obtained were then corrected manually for obvious misalignments. Sequence alignment for the introns within LW Rh was trivial and required only three simple gaps, which were parsimony-uninformative. However, alignment for introns within ArgK and EF-1 $\alpha$  required gaps of various lengths, which differed markedly in terms of structural characteristics; relatively long gaps that were easily aligned occurred throughout ArgK intron sequences (fig. 1A), whereas numerous shorter gaps were required at five particular regions within EF-1 $\alpha$  intron sequences, resulting in multiple optimal alignments (fig. 1B). This difference in gap characteristics provided an excellent opportunity to study different evolutionary modes of length mutations occurring within the diverse genus *Bombus*.

All phylogenetic analyses were done using PAUP\* version 4.0b10 (Swofford 2002). Many intron sequences of the outgroup, *Trigona ventralis*, were highly dissimilar to those of the ingroup. We therefore included only unambiguously determined regions (corresponding to 42% of the intron alignment) for the outgroup in the analysis. The results of partition-homogeneity test (Farris et al. 1994), as implemented in PAUP\*, suggested that phylogenetic signals within alignment-unambiguous regions of the three

genes were highly congruent ( $P > 0.5$  in 999 random partitioning for all data comparisons). We then performed a simultaneous analysis of the data set of substitution characters from all unambiguously aligned regions (with gaps treated as missing) to obtain a robust species relationship—the test phylogeny—using the maximum parsimony (MP) method. We conducted heuristic searches with 100 random addition analyses and tree bisection-reconnection (TBR) branch-swapping (Steepest descent option in effect). In order to assess the robustness of the MP tree to the use of explicit models of sequence evolution, we also performed neighbor joining (NJ) and maximum likelihood (ML) analyses. We used the HKY85 model for distance correction in the NJ analysis. To search for a ML tree, we used the quartet puzzling option as implemented in PAUP\* with the HKY85+ $\gamma$  substitution model.

We investigated the influence of gap characters on phylogenetic inference by conducting an additional parsimony analysis with gap information included as coded characters. Alignable gaps of ArgK intron were coded as binary (presence/absence) characters using the method of Simmons and Ochoterena (2000) and added to the data matrix. We weighted base substitutions and indel mutations equally, because there was no a priori reason to differentially weight gap costs relative to base substitutions. For the ambiguously aligned regions of EF-1 $\alpha$  intron (fig. 1B), we employed the program INAASE (Integration of Ambiguously Aligned Sequences) (Lutzoni et al. 2000) to accommodate these regions in parsimony analysis. Following the criteria detailed in Lutzoni et al. (2000), we delimited five ambiguously aligned regions within the EF-1 $\alpha$  intron. However, coding one of these regions (region 4) resulted in more than 32 multistate characters, which exceeds the number that can be handled by PAUP\*. We therefore divided the region into two subregions such that sequences within each region are likely homologous (region 4a and region 4b in fig. 1B). Several division schemes that we tried all yielded the same number of most-parsimonious trees of the same topology, indicating the validity of this approach.

## Results and Discussion

Parsimony analysis of the unambiguously aligned region (2301 characters with 395 informative) resulted in 891 most-parsimonious trees of length 1338 (consistency index excluding uninformative characters [CI] = 0.47, retention index [RI] = 0.76, and rescaled consistency index [RC] = 0.45). The topology obtained (fig. 2) is, with

FIG. 1.—A, Partial alignment of ArgK intron. Parsimony-informative gaps, treated as single indel mutations, are indicated by ===. *B. nevadensis* had an insertion of 323 bp replacing an 111–122 bp sequence present in other species. This sequence was apparently unrelated to the sequences at corresponding positions in other species, so we excluded the sequence and coded it as inapplicable in the alignment (poly-n). Similarly, *B. mendax* and *B. defector* shared identical 46 bp sequences that were ambiguously aligned with respect to other sequences of corresponding position (shaded sequences). Because inclusion of these sequences affected inference of indels in other species, we ignored these sequences in the alignment and instead interpreted their origin as a single insertion event (gap-MD). Only sequences that account for informative gaps are given, and only regions that contain these gaps are shown (separated by vertical bars). Numbers above sequences are positions within aligned intron sequence for the first and last codons of each segment. B, Partial alignment of EF-1 $\alpha$  showing the entire intron sequences. Ambiguously aligned regions are indicated by ===. Examples of different delimitations that we tried are given for region 4 (a, b); the bottommost scheme was used in the analysis. Only sequences that account for alignment ambiguity are shown.



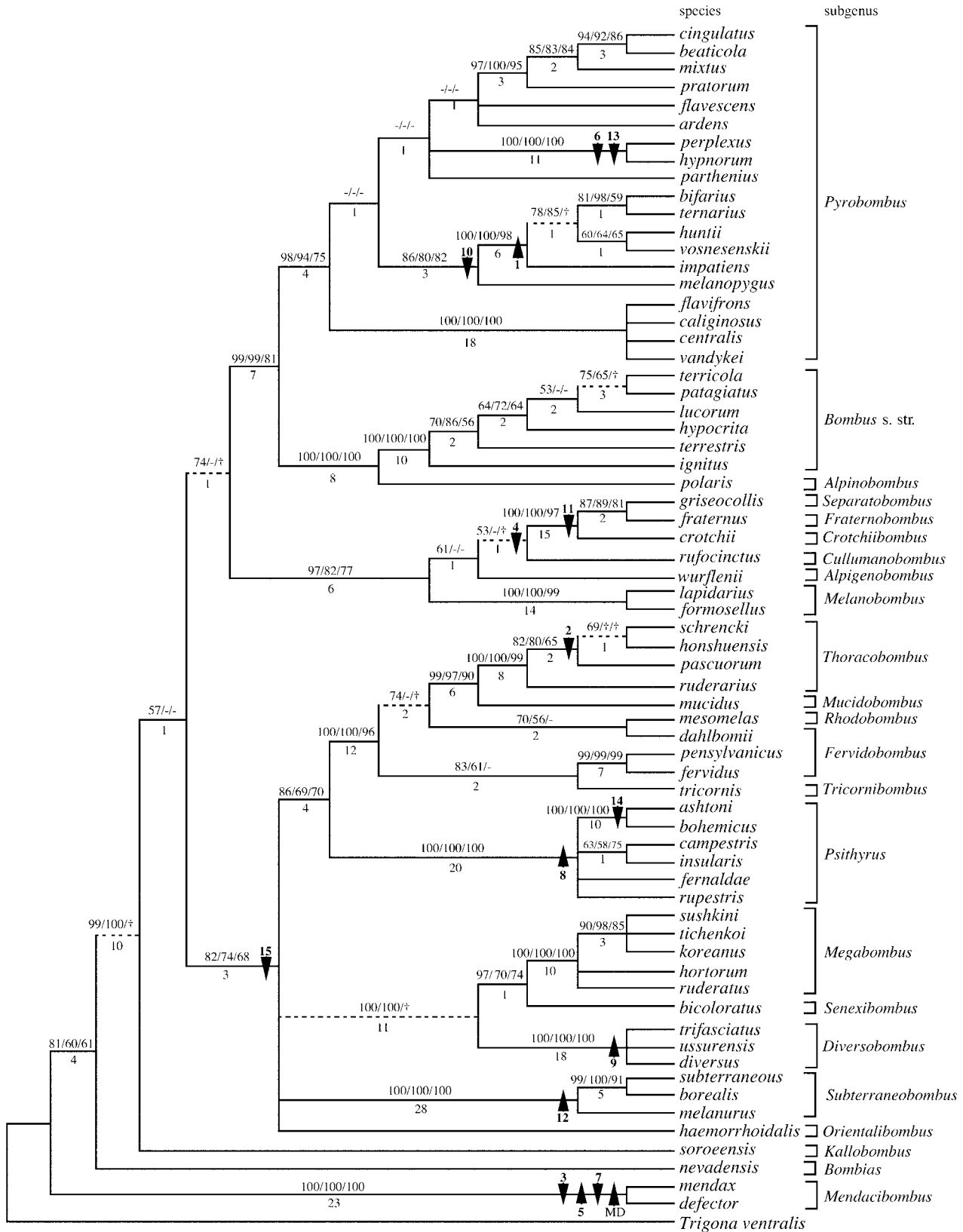


FIG. 2.—Strict consensus of 891 most-parsimonious (MP) trees based upon base substitutions of the unambiguously aligned regions. The 16 unambiguously aligned indels within ArgK intron were mapped onto specific branches of the tree without homoplasy. Upward and downward arrowheads represent insertions and deletions respectively. Numbers on arrowheads correspond to the gap codes in Fig. 1A. Branches that collapse in the NJ and/or ML trees are presented as dotted lines. Nodal support is assessed by bootstrap values (above branches; MP/NJ/ML respectively; shown only when >50%) and branch support indices (below branches).

few exceptions, consistent with traditional subgeneric classifications of bumble bees based on adult morphological characters (Williams 1998). The topologies of NJ and ML trees are similar to that of MP tree (see supplementary online materials).

Overall, we inferred 16 parsimony-informative gaps within the ArgK intron (fig. 1A). All gaps were successfully assigned to specific branches of the MP tree without homoplasy (fig. 2), suggesting that the signal of gaps is concordant with that of base substitutions. This result holds for the NJ and ML trees with one exception. On the MP tree gap-4 was a synapomorphy uniting the species *B. griseocollis*, *B. fraternus*, *B. crotchii*, and *B. rufocinctus*, but monophyly of these four species was not recovered on the ML tree due to the sister relationship of *B. rufocinctus* to a species that lacks gap-4, *B. wurflenii* (fig. 2). Support in the ML analysis for paraphyly of the four species with gap-4 was ambiguous, as demonstrated by the extremely short lengths of the branches concerned. Therefore congruence of gap-4 with the phylogeny is not strongly rejected.

Analysis of the data matrix including the 16 characters coding ArgK gaps ("GAP" characters throughout) resulted in the same shortest trees (1354 steps: CI = 0.48; RI = 0.77; RC = 0.46) as did the data matrix including only base substitutions. Inclusion of GAP characters (16 of 411 informative characters) did not contribute to resolution, but bootstrap values for the nodes concerned increased from >53% to >72%, and the total branch support (Bremer 1994) increased from 323 to 335.

The six multistate characters coding ambiguous regions of EF-1 $\alpha$  ("INAASE characters" throughout) mapped onto the substitution-based tree with a total of 187 steps and a CI of 0.77 (RI = 0.87; RC = 0.67). This indicates that the INAASE characters contain rich information consistent with the phylogenetic signal of base substitutions. Simultaneous analysis of an expanded data matrix, including the 16 GAP characters and the six INAASE characters resulted in four most-parsimonious trees of length 1535 (CI = 0.53; RI = 0.78; RC = 0.48) (supplementary online data) with lower homoplasy levels than the analysis based on substitutions and GAP characters. The six INAASE characters collectively had CI = 0.80, RI = 0.89, and RC = 0.71. Although the alignment ambiguous EF-1 $\alpha$  intron regions accounted for only ~5% of all sites in the original data matrix, INAASE characters added 181 steps to the tree (12% of the total steps) and contributed substantially to phylogenetic resolution, resulting in a drastic decrease in the number of shortest trees (from 891 to 4) and recovery of 62 of 64 possible nodes in the strict consensus tree (an addition of 9 nodes). INAASE characters also significantly improved nodal support, increasing average bootstrap values by 3.9% (based on nodes in common on the strict consensus trees), and summed branch support by 55 (from 335 to 390).

Our analyses showed that gaps and ambiguously aligned regions of nuclear intron sequences contain useful phylogenetic signal concordant with that of base substitutions. Unambiguously aligned gaps exhibited minimal homoplasy and were consistently congruent with the substitution-based tree, whether derived from MP, NJ, or ML method. Thus, our results reinforce several earlier

suggestions that gaps are phylogenetically reliable characters (Lloyd and Calder 1991; van Ham et al. 1994; Graham et al. 2000; Simmons, Ochoterena, and Carr 2001). For example, Lloyd and Calder (1991) found that all seven informative gaps longer than 1 bp within  $\psi\eta$ -globin pseudogene of primates could be assigned to specific branches of substitution-based tree without homoplasy. Similarly, van Ham et al. (1994) showed that 13 of 15 informative gaps longer than 1 bp could be mapped on substitution-based *trnL-trnF* intergenic spacer gene tree in Crassulaceae plants without homoplasy. However, in these studies single-nucleotide indels were often homoplastic or ambiguously aligned. Multinucleotide indels are probably more reliable than single-nucleotide indels because the former are less frequent than single-nucleotide indels in many data sets (Saitou and Ueda 1994; van Ham et al. 1994; Graham et al. 2000) and because homoplasies by parallel and back mutations can occur only when they match exactly in length and position (and sequence for insertions) with the corresponding indels (Lloyd and Calder 1991).

On the other hand, the results of several other studies contrast with those described above. For example, in Baptiste and Philippe's (2002) analysis of eukaryotic phylogeny, the observed pattern of amino acid indels within enolase and *impdh* genes likely resulted from recombination and lateral gene transfer as well as from convergence and reversal. In other cases, apparently identical indels seem to have originated independently in distantly related taxa (van Ham et al. 1994; Philippe and Laurent 1998; Graham et al. 2000). However, these results do not necessarily indicate that indels are generally of lesser utility than base substitutions, because homoplasy can be found in any class of molecular characters, especially when dealing with phylogeny on a large scale (e.g., across phyla). Considering these results together with ours, we conclude that indels can be highly reliable characters, especially at lower taxonomic levels, but recognize that gaps, like all classes of phylogenetic characters, are not devoid of homoplasy. It may therefore be inadvisable to identify higher monophyletic groups based solely on a single indel (or a few indels) (Baptiste and Philippe 2002).

Recent methodological progress in handling gap characters in phylogenetic analyses affords the opportunity to incorporate this useful phylogenetic information derived from sequence data. However, these new methods are confined to the parsimony optimality criterion, and a well-justified, statistically-robust, generally applicable, and widely accepted method for incorporating indels within explicitly model-based phylogenetic methods such as standard implementations of ML is still lacking. The development of explicit models of indel evolution should not be particularly difficult, but selection of parameters to be included and statistical interpretation of resulting analyses will be far from straightforward (see Farris 1999; Sanderson and Kim 2000). Even within the context of parsimony analysis, the difficulty of assuming gap-to-substitution costs or weighting gaps of different lengths has impeded extensive use of the methods. However, gaps are strong indicators of common descent, and full

utilization of indel information to corroborate and refine phylogenies inferred primarily from substitution data would surely improve the accuracy and efficiency of phylogeny estimation for many data sets (e.g., Rokas and Holland 2000; Danforth 2002).

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