Molecular systematics and biogeography of *Nicrophorus* in part—The investigator species group (Coleoptera: Silphidae) using mixture model MCMC

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Burying beetles (Silphidae: *Nicrophorus*) are well-known for their biparental care and monopolization of small vertebrate carcasses in subterranean crypts. They have been the focus of intense behavioral ecological research since the 1980s yet no thorough phylogenetic estimate for the group exists. The relationships among the species, and the validity of some species, are poorly understood. Here, we infer the relationships and examine species boundaries among 50 individuals representing 15 species, primarily of the investigator species group, using a mixture-model Bayesian analysis. Two mitochondrial genes, COI and COII, were used, providing 2129 aligned nucleotides (567 parsimony-informative). The Akaike Information Criterion and Bayes Factors were used to select the best fitting model, in addition to Reversible Jump MCMC, which accommodated model uncertainty. A 21 parameter, three-partition GTR + G was the final model chosen. Despite a presumed Old World origin for the genus itself, the basal lineages and immediate outgroups of the investigator species group are New World species. Bayesian methods reconstruct the common ancestor of the investigator species group as New World and imply one later transition to the Old World with two return transitions to the New World. Prior hypotheses concerning the questionable validity of four species names, *Nicrophorus praedator*, *Nicrophorus confusus*, *Nicrophorus encaustus* and *Nicrophorus mexicanus* were tested. No evidence was found for the validity of the *Nicrophorus investigator* synonym *N. praedator*. We found evidence rejecting the species status of *N. confusus* (NEW SYNONYM of *Nicrophorus sepitor*), *N. encaustus* and *N. mexicanus*, which are tentatively retained as valid. Our results strongly reject a recently published hypothesis that *Nicrophorus interruptus* (NEW STATUS as valid species) is a subspecies of *N. investigator*.

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1. Introduction

There is a clear and well-justified increase in concern with the process of phylogenetic model selection, particularly for large, multi-gene datasets (Alfaro and Huelsenbeck, 2006; Posada and Buckley, 2004). In Bayesian analyses, to a greater degree than in maximum-likelihood analyses, it appears there are real dangers associated with use of too-simple models (Huelsenbeck and Rannala, 2004). As dataset complexity has grown, the trend has been to fit multiple models to gene partitions established *a priori*. This is certainly a way to avoid model underfitting, and a clear improvement over the use of a single model, the assumptions of which (e.g. data homogeneity) would be dramatically violated (Brandley et al., 2005). However, a new problem results—how to best partition the data? For large multi-gene datasets there could be hundreds of possible partitions to evaluate and model-fit prior to analysis. A solution to this problem may be found in the development of mixture-model approaches like those of Pagel and Meade (2004, 2005) which allow the number of distinct patterns in the data to be found during analyses, without the need for a priori partitioning and model-fitting. This new approach was employed and evaluated by Collins et al. (2006) for a two gene dataset analysis of medusozoa who, despite the apparent advantages of mixture-modeling, concluded it was “of little additional value over a more traditional phylogenetic approach.” The aims of the present study are to further explore the mixture-model approach of Pagel and Meade (2004, 2005) and to explicitly test hypotheses of species boundaries and infer relationships for species of the investigator group in the genus *Nicrophorus* (Coleoptera: Silphidae).

Beetles of the genus *Nicrophorus* Fabricius 1775, (Silphidae: Nicrophorinae), commonly called burying beetles, are among the better-known insect lineages. The ease of manipulation in the field and laboratory has made them model organisms for studies in ecology, physiology and behavior. The ability to transport and bury a small vertebrate carcass, to remove hair or feathers from the carcass and then to round it into a brood ball was the first burying
beetle behavior to receive intensive study (Fabre, 1899; Milne and Milne, 1944). Early work on competition for resources and parental care (Pukowski, 1933) was the foundation for over 150 behavioral ecology studies in the past 25 years. Study of biparental care has focused on why the second parent stays to provide care (Bartlett, 1988; Eggert and Sakaluk, 1995; Jenkins et al., 2000; Koulianos and Schwarz, 2000; Müller et al., 1998; Müller and Eggert, 1989; Sakaluk et al., 1998; Satou et al., 2001; Scott, 1989, 1990, 1994; Scott and Gladstein, 1993; Trumbo, 1991, 2006), how a partner is chosen (Rauter and Moore, 2004; Smiseth and Moore, 2003), and how conflicts over care are resolved (Rauter and Moore, 2004; Smiseth and Moore, 2004).

Ecological studies of burying beetles have focused on whether or not symbiotic phoretic mites of the genus *Poecilocliths* benefit or harm their burying beetle hosts (Beninger, 1993; Blackman, 1997; Schwarz et al., 1998; Wilson, 1983; Wilson and Knollenberg, 1987), on seasonal effects on competition and life history (Anderson, 1982b; Ohkawara et al., 1998; Meierhofer et al., 1999; Nagano and Suzuki, 2003; Nismura et al., 2002; Smith and Merrick, 2001; Trumbo and Bloch, 2002; Wilson et al., 1984), and on interspecific takeovers of carcasses (Eggert and Sakaluk, 2000; Noble and Noble, 2004). A compelling group to study comprehensive attention since the world revisions of Portevin (1920, 1971; Suzuki, 2000, 2004; Trumbo, 1990).

Good summaries of this literature on the behavior and ecology of burying beetles have been written by Ratcliffe (1996), Trumbo (1996), Eggert and Müller (1997) and Scott (1998). The extensive use of burying beetles as research models and intensive study of the endangered American burying beetle have produced a wealth of natural history information on the distribution, habitat preferences, phenology and diel periodicity, especially for North American, European and Japanese species (Anderson, 1982a; Bedick et al., 1999; Creighton et al., 1993; Holloway and Schnell, 1997; Kozol et al., 1988, 1994; Lomolino et al., 1995; Lomolino and Creighton, 1996; Sikes and Raithel, 2002). The availability of data on behavior and ecology make *Nicrophorus* a compelling group to analyze life history traits from a phylogenetic perspective.

The 21 New World species, including the most recently described New World species, *Nicrophorus hispaniola*, have been revised in modern times (Anderson and Peck, 1985; Peck and Anderson, 1985; Sikes and Peck, 2000). However, the majority of species (40+) are northern-temperate Old World, and have seen little comprehensive attention since the world revisions of Portevin (1926), Hatch (1927), and Semenov-Tian-Shanski (1933). Sixty-one extant, valid species in the genus *Nicrophorus* were cataloged by Sikes et al. (2002) in the most recent taxonomic work on the group that was global in scope. Seven new species were described in, and a phylogenetic analysis conducted of, the *nepalensis* group, the second largest species group in the genus by Sikes et al. (2006).

In the current paper we focus on inference of the phylogeny of 11 of the 16 currently valid species in the largest species group in the genus—the investigator species-group (Table 1) sensu Sikes (2003). To date, seven modern phylogenetic studies have been published that include *Nicrophorus* species. Peck and Anderson (1985) revised the New World species and these authors’ cladistic analysis, based on adult and larval morphology, and ecology, placed 20 New World species into four species-groups (Fig. 1) with two unplaced species—concluding the New World fauna did not form a monophyletic group. A manually calculated cladistic analysis of the Korean fauna was performed by Cho et al. (1988). Unfortunately, Cho et al.’s dataset was based on superficially evaluated characters and lacked resolving power due to a dearth of informative characters. Růžička (1992) applied parsimony methods to a carefully constructed dataset based on larval characters of five central European species and was able to place four of these species into three of Peck and Anderson’s (1985) species groups. Palestrini et al. (1996) described the larva of *Nicrophorus mexicanus* and presented the first phylogenetic assessment focused entirely on the investigator species group. Unfortunately, their work lacked both sufficient taxa (only 6 of 16 species were included) and characters (4 parsimony informative) to be of much value. The first molecular phylogenetic investigation of the family Silphidae, which focused on the intergeneric relationships of the subfamily Silphinae, was conducted by Dobler and Müller (2000). This work demonstrated that the gene regions COI and COII contain sufficient information for phylogenetic resolution of generic and specific relationships within this family. The same year Szalanski et al. (2000) published a small phylogeny including nine *Nicrophorus* species, four of which are included in the present paper. The most recent phylogenetic work in the family is that of Khatchikov and Popov (2006) who proposed a number of taxonomic changes based on study of the male and female genitalia. Most relevant to our study was their conclusion that *Nicrophorus interruptus* Stephens is a subspecies of *N. investigator* Zetterstedt.

### 1.1. The investigator species group

The investigator species group (Table 1 and Fig. 2) sensu Sikes (2003) includes members of the groups Hatch (1927) named the *pustulatus* group (seven valid species) and the *marginatus* group...
Hatch (1927) concluded that most Asian members of the genus *Nicrophorus* were primitive. Peck and Anderson (1985), Newton (1997), Dobler and Müller (2000), and Sikes (2003) agreed the genus *Nicrophorus* was probably Eurasian in origin because the closely related microphorine genera, *Promascopus* and *Eonecrophorus*, are Asian and because more species of *Nicrophorus* occur in Eurasia than in the New World. Peck and Anderson's (1985) cladistic analysis of the New World fauna resulted in four species groups and two unplaced species (Fig. 1). They hypothesized at least six ancestral dispersal events from the Old World to the New, one for each of the four species groups and two for each of the unplaced species. They suggested these occurred during the Tertiary or the Pleistocene. Using maximum-likelihood ancestral state reconstruction techniques Sikes (2003) concluded, with 93% confidence, that the ancestor of the genus *Nicrophorus* was Laurasian but was not able to statistically distinguish an Old versus New World origin due to lack of phylogenetic resolution.

Regarding the *investigator* species group specifically there have been no hypotheses put forth regarding the group’s biogeographic history. However, Peck and Anderson’s (1985) work suggests the New World species are recent and derived relative to Old World species. Based on the relationships shown in their cladogram of the *investigator* species group (Fig. 1) biogeographic reconstructions could range from one ancestral invasion of the New World at the root with a single return invasion of the species *N. investigator*—making the basal lineages of the group descendants of New World ancestors (New World species form a monophyletic group), to a scenario in which three separate invasions of the New World occurred with no reversals (polyphyletic New World species). An intermediate possibility is a New World invasion at the root, then a return invasion of the Old World leading to *N. investigator* (and possibly other Old World species) with a third transition of this derived Holarctic species to reinvade the New World (paraphyletic New World species). Questions we hope to answer include “Do the New World species form a monophyletic group (one invasion with no reversal), a paraphyletic group (one invasion with reversal) or a polyphyletic group (multiple invasions with or without reversals)?”

### 1.3. Questionable *investigator* group species

Most traditionally defined species hypotheses have yet to be explicitly tested. Although there is little agreement on which, if any, method is best to define species there is a general consensus that formal, explicit tests should be conducted (Johnson et al., 2004; Robins et al., 2006; Sites and Crandall, 1997; Sites and Marshall, 2003; Wiens and Penkrot, 2002). The *investigator* species group includes the greatest number of questionable species hypotheses in the genus. Most of these have been dealt with or commented on in the taxonomic catalog of Sikes et al. (2002). For this study we hoped to investigate more closely the validity of four names: *Nicrophorus confusus* Portevin, *Nicrophorus encaustus* Fairmaire, *N. mexicanus* Matthews, and *Nicrophorus praedator* (Reitter). Unlike many insect taxa, structures of the genitalia of either sex are not useful to differentiate closely related *Nicrophorus* species, (with very few exceptions)—as is true for all species of the *investigator* group (Sikes, 2003).

*Nicrophorus confusus* is unquestionably distinct from *Nicrophorus sepultor* Charpentier. They are purportedly diagnosable based on the color of the setae of the posterior margin of the metasternum (as described in Sikes et al. (2002)). However, the examination of 256 specimens of *N. sepultor* from throughout its range and 32 specimens of the much rarer *N. confusus* suggests these may represent the same, but somewhat polymorphic, species (Sikes et al., 2002; Sikes, 2003).

Between the sister species of the two pairs (*N. mexicanus* + *N. nigrita* Mannerheim) and (*N. encaustus* + *N. investigator* Zetterstedt)—both showed very small genetic divergences in preliminary analyses, differ only by color characteristics, and have parapatric distributions. We hoped to test the monophyly of each species with a phylogenetic analysis based on samples of their mitochondrial DNA sequences.

A collaborator of ours, R. Madge, has studied the taxonomy of the genus *Nicrophorus* for many decades. He considered the name *N. praedator* valid and belonging to a species that could be differentiated, albeit with difficulty, from *N. investigator* by the shape of the pronotum of large males. He concluded (unpublished) that in the larger males of these species the pronotum appeared to be less transverse in what he considered *N. praedator* than in *N. investigator*. We chose to investigate this hypothesis, which, if correct, should allow one to distinguish at least the larger males of *N. praedator* from those of *N. investigator*. Although *N. praedator* was synonymized in 2002 we considered

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**Fig. 1.** Phylogeny of the New World species (minus *N. hispaniola* Sikes and Peck), after Peck and Anderson (1985) showing four species groups and two unplaced species. The New World *investigator* group species are indicated by the asterisk.
its existence, as a cryptic species, probable enough to warrant detailed study. For reference, we also measured the species N. interruptus, which is hypothesized to show similar pronotum characteristics to N. praedator (R. Madge, in litt.).
In addition, we hoped to identify a lineage corresponding to *N. praedator* by sequencing mtDNA from specimens collected within the supposed distribution of *N. praedator*. If *N. praedator* is valid it would often be mis-identified as *N. investigator*, suggesting it might be a closely related species. It would be a strong result if we found evidence of *N. praedator* but a weak result if we find none (because demonstrating something does not exist is far more difficult than the opposite).

We apply the species delimitation methods described by Wiens and Penkrot (2002) in which distinct species should, given sufficient time since divergence, have strongly supported and exclusive (monophyletic) haplotype phylogenies relative to other species; have one or more diagnostic morphological characters (either fixed or at high frequency); and form strongly supported clades of populations based on morphology. The last criterion requires a morphology-based phylogeny incorporating multiple samples per species—a step we did not undertake. Instead, we conducted a morphometric analysis to help identify the questionable species *N. praedator*.

Our phylogenetic analysis provided us the opportunity to test these species hypotheses, compare our results to prior cladistic analyses at 94 °C, and an a priori partitioned analysis. These approaches to Bayesian phylogenetic inference help overcome the limitations of assuming a single homogenous model of substitution applies to all the data.

2. Materials and methods

2.1. Taxon sampling

Eleven species of the 16 known to belong to the *investigator* group were obtained, many by the generous efforts of collaborators, for sequencing (Table 1 and Appendix A). Typically, specimens were obtained by use of hanging or pitfall traps baited with rotten meat (chicken or fish). Living adults were placed into 15 ml vials of 95–100% ethanol in the field, one beetle per vial with a data label, and their hind legs were separated from their bodies to help the ethanol quickly penetrate and preserve the muscle tissues of the hind legs. These specimens were later database at the University of Calgary, (Faculty of Medicine, University of Calgary, Calgary, AB).

Preliminary, unpublished, results for the entire subfamily Nicrophoridae indicated the *investigator* species group is monophyletic based on both morphological data and COII sequences (Sikes, 2003). These results also indicated that the *marginatus* species group is the sister clade to the *investigator* group—making it an obvious choice as an outgroup. Slightly less close, but nevertheless near, the *investigator* and *marginatus* groups was the *vespilloides* group, which we chose to root our trees. The final dataset includes 50 sequences, eight of which represent outgroup taxa. Effort was made to sequence multiple individuals preferably from different populations of each species to test hypotheses of species monophyly. Due to the rarity of numerous species in this group and the difficulty of obtaining preserved tissues we did not obtain samples from all known species but were able to obtain samples for the questionable species we wanted to investigate. The most widespread species in the group, *N. investigator*, which is Holarctic, was the most thoroughly sampled. For this species, we obtained samples of both New World populations and various Old World populations in the regions of the supposed distribution of *N. praedator* (Japan: Honshu, Japan: Hokkaido, northeast China, eastern Russia).

Identifications were obtained by use of morphological characters and species descriptions in the keys of Sikes (2003) and Anderson and Peck (1985). Bodies of these DNA voucher specimens are stored in 95–100% propylene glycol at −70 °C in the collection of the senior author.

2.2. DNA extraction, amplification, and sequencing

Complete genomic DNA was extracted from the hind-leg muscle tissue of each absolute ethanol-preserved specimen (Appendix A) and stored at −80 °C using the Qiagen DNeasy® kit. Extraction success was confirmed visually on an agarose gel stained with ethidium bromide. Amplification and sequencing of the COI region was accomplished using three primer pairs. The first two pairs covered the 5′-half of the COI gene: TY-J-1460 (5′-TAC ATT TTA CCT CAA CTG CAG C-3′) and C1-N-2191 (alias ‘Nancy’) (5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′) in addition to C1-J-1718 (5′-GGA GGA TTT GGA AAT TGA TTA TTC CC-3′) and C1-N-2329 (alias ‘K525′) (5′-ACT GTA ATT ATA TGA CCT CA-3′). Typically, the first set was used because it amplifies a larger section and is very reliable. The 3′-half of COI was amplified using the following primer pair: C1-J-2195 (5′-TTG ATT TTT TGG TGA TCC AGA AGT-3′) and TL2-N-3014 (alias ‘Pat’) (5′-TCC AAT GCA CAT TGC TGC ATT A-3′). Amplification and sequencing of the mitochondrial COII gene was accomplished with the primers TL2-J-3034 (5′-ATG GCA GAT TAG TGC A-3′) and A8-N-3914 (3′-TCA TAT TAT TGG TGA TAT TGG AGG-5′) (Simon et al., 1994).

Each 50-±μL PCR cocktail contained 2 μl of template DNA and 48 μl of master mix (which was comprised of 1 μl of dNTP 5 μl of 10× TAE buffer, 2.5 μl of forward primer, 2.5 μl of reverse primer, 37 μl of deionized water, and 0.25 μl of Tag). Typical amplification was accomplished via an initial 3-min denaturation step at 94 °C, and 29–35 subsequent iterations of the following cycle: 30 s denaturation at 94 °C, 1 min annealing at 47 °C, and 1 min elongation at 72 °C. A 10-min elongation at 72 °C terminated the reaction. Amplified PCR products were subsequently purified according to the protocol provided in the Qiagen QIAquick® Spin Handbook (Mississauga, ON, Canada). Final sequences were obtained from an automated 3730 ABI DNA Analyzer at the DNA Core Services Center at The University of Calgary and the University of Tokyo (Faculty of Medicine, University of Tokyo, Japan).

2.3. DNA sequence editing and alignment

Each gene region was bidirectionally sequenced to verify accuracy. These sequence data were assembled and aligned with each other to create a single consensus sequence using the software Sequencher v 3.0 (Gene Codes Corp.; http://www.gene codes.com) and/or CodonCode Aligner v1.2.0 (CodonCode Corporation, Dedham, MA; http://www.codoncode.com). Data were aligned by eye with reference to codon position and amino acid sequence based on the protein-coding sequence. Minor length variation was seen in the tRNA sequences adjacent to COII.

2.4. Data partition congruence

To ascertain if the signal in the two genes, COI and COII, was significantly different we employed the partition homogeneity test (aka the incongruence length difference (ILD) test, Farris et al., 1994) in PAUP* 4.0b10 using 100 replicates each based on a single random addition sequence starting tree swapped to completion via TBR (the COI partition included the adjacent tRNAs). Because this
test has been criticized for providing both false negatives and false positives under certain circumstances (Barker and Lutzoni, 2002; Ramirez, 2006) we tested maximum likelihood topologies preferred by each partition separately and the combined data against the other datasets using the Shimodaira–Hasegawa (Shimodaira and Hasegawa, 1999) test.

Maximum-likelihood trees for the SH test were found using an iterative approach (Sullivan et al., 2005) for each partition separately and the combined data as follows: the best fitting model as determined by the AIC, which was GTR + I + G for all partitions, was used in all cases. The parameters provided by MrModeltest v2.2 (Nylander, 2004) for each dataset were fixed for 1000–30,000 rounds of TBR branch swapping on a starting tree obtained by neighbor-joining. The resulting topology was used to estimate a new set of parameters which were then fixed for a subsequent search. This was repeated until parameter values, the topology, and the –lnL no longer changed. The best trees for each partition and the combined data were then compared to one another under a 3Q GTR + I + G model (see ‘Model Selection’ below) with the program MrBayesPhylogenies. The 90% majority rule consensus topologies resulting from each individual partition were then compared visually to determine if any strongly-supported but contradictory branches were present. This last approach accommodates uncertainty in the data—only well-supported (>90%PP) branches are considered data partitions resulting in the fit of 24 models evaluated (see ‘Model Selection’ below) with the program MrBayesPhylogenies (Pagel and Meade, 2006; Posada and Buckley, 2004). We used the program MrModeltest v2.2 (Nylander, 2004) to evaluate the fit of 24 models with more rate matrices. Traditional a priori partitioning is a special, restricted, case of mixture-modeling in which some sites in the data are assigned a matrix weight (w) of zero (Pagel and Meade, 2005). This is an extreme form of model-fitting which may be justified for some data types (e.g. morphology vs molecular) but for many types it may be impossible to be certain a priori which model(s) best fit which sites. Mixture modeling allows multiple models to apply with some probability to each site—thereby better accommodating uncertainty than traditional partitioning (Pagel and Meade, 2005). Matrix weights are interpreted as follows (Pagel and Meade, 2005): the data at a given site arose with the probability specified by the weight from the model implied by the rate parameters of that matrix. For example, one matrix might describe the pattern of evolution that tends to predominate at coding positions, while another may fit the pattern seen in ribosomal stem positions, but both matrices are applied with some probability to every site.

However, adding rate matrices increases the number of parameters being estimated. For example, the GTR + G model requires six rate parameters plus one weight parameter thus requiring seven additional parameters estimated for each additional matrix. Therefore, at some point while adding matrices to an analysis the model will become too complex (not worth the added parameters) which will be evidenced by only slight improvements of the log-likelihood score (e.g. Pagel and Meade, 2005, Fig. 1.3) and low (near zero) matrix weights for added matrices.

In our case, based on the MrModelTest results reported above, we used the most complex model available, GTR + G, (a parameter for invariant sites is not available in this program because rate heterogeneity of this nature would be accommodated by additional rate matrices, if necessary) and compared the fit using 1 through 4 rate matrices (Table 3). Each model was evaluated using a run composed of three or four MCMC chains (1 cold, 2–3 heated) sampled once every 1000 steps and run for 2 million steps (models 1, 2 and 4Q) or 5 million steps (models 3Q, 3UV) for invariable sites (tRNAs). We ran this analysis twice for 2 million steps sampling the cold chain once every 1000 steps. These two analyses were compared for convergence using Gelman and Rubin’s potential scale reduction factor (Gelman, 1996; Gelman and Rubin, 1992a, 1992b) as implemented in MrBayes, which converged on 1 for all parameters indicating convergence had been reached. We also performed a single model GTR + I + G nonpartitioned analysis. The mean of the harmonic means of the log-likelihoods of the nonpartitioned runs was –10,800, which is more than 170 log-likelihood units worse than the 3Q GTR + G mixture model.

The mean of the harmonic means of the log-likelihoods of the a priori partitioned analyses was –10,368 (Table 3) which is over 200 log-likelihood units better than the best BayesPhylogenies,

<table>
<thead>
<tr>
<th>Model</th>
<th>Log-likelihood</th>
<th>lnL mean difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC</td>
<td>–12683.3252</td>
<td>–11704.4795</td>
</tr>
<tr>
<td>F81</td>
<td>–12454.3576</td>
<td>–11385.3594</td>
</tr>
<tr>
<td>K80</td>
<td>–12538.0791</td>
<td>–11356.4902</td>
</tr>
<tr>
<td>SYM</td>
<td>–11944.7324</td>
<td>–10304.7617</td>
</tr>
<tr>
<td>GTR</td>
<td>–11817.0098</td>
<td>–10755.5020</td>
</tr>
</tbody>
</table>
three pattern, analysis (−10,626). We compare the results of the MrBayes and the BayesPhylogenies analyses below.

Pagel and Meade (2005) describe a “rule of thumb” for the cost of adding rate matrices within a Bayes Factor framework for model selection. They demonstrate that with the GTR + G model a score would have to be 70–80 or more log-likelihood units greater (Pagel and Meade, 2004) to make the extra rate matrix worth the added parameters. We chose the three rate matrix model because it returned a score 75 log-likelihood units greater than a two rate matrix model. Our third run using the three rate matrix model did not reach stationarity until step 2.3 million of the 5-million step run (Fig. 3). The addition of a fourth rate matrix did not significantly improve the log-likelihood, indicating the additional matrix was redundant.

Table 3
Harmonic means of marginal log-likelihood scores and mean tree lengths (TL) using multiple rate matrices in BayesPhylogenies (non-RJ version) for the analyses of the combined COI and COII data compared with the results of MrBayes (GTR + G, three partitions: codons 1 and 2, codon 3, noncoding).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Run 1 (ESS)</th>
<th>Run 2 (ESS)</th>
<th>Run 3 (ESS)</th>
<th>Mean (ESS)</th>
<th>Difference</th>
<th>Mean TL (ESS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1Q GTR</td>
<td>−11,867 (205)</td>
<td>−11,867 (326)</td>
<td>−11,867 (163)</td>
<td>−11,867 (694)</td>
<td>−</td>
<td>0.823 (429)</td>
</tr>
<tr>
<td>2Q GTR</td>
<td>−10,702 (188)</td>
<td>−10,702 (212)</td>
<td>−10,701 (291)</td>
<td>−10,701 (690)</td>
<td>108</td>
<td>1.272 (125)</td>
</tr>
<tr>
<td>3Q GTR</td>
<td>−10,626 (443)</td>
<td>−10,624 (627)</td>
<td>−10,630 (241)</td>
<td>−10,626 (1310)</td>
<td>75</td>
<td>1.346 (105)</td>
</tr>
<tr>
<td>4Q GTR</td>
<td>−10,630 (148)</td>
<td>−10,598 (46)</td>
<td>−10,604 (148)</td>
<td>−10,600 (513)</td>
<td>26</td>
<td>1.470 (81)</td>
</tr>
<tr>
<td>3P GTR</td>
<td>−10,369 (240)</td>
<td>−10,368 (272)</td>
<td>−10,367 (314)</td>
<td>−10,368 (799)</td>
<td>232</td>
<td>2.159 (419)</td>
</tr>
</tbody>
</table>

The difference in log-likelihood units is listed between the model of that row and the model one row above. Effective sample sizes, as calculated by the program Tracer v1.3 (Rambaut and Drummond, 2003) are listed in parentheses. Total values were obtained by simultaneous analysis of three post burn-in samples using Tracer. Chosen model is in bold.

2.6. Stationarity

There are a variety of methods to assess if an MCMC analysis has reached stationarity (e.g. Drummond et al., 2002; Lewis and Lewis, 2005) although no method can prove convergence has been reached. For the MrBayes analyses we evaluated convergence using the potential scale reduction factor (Gelman, 1996; Gelman and Rubin, 1992a, 1992b) as implemented in MrBayes, which converged on 1 for all parameters indicating convergence had been reached.

For the BayesPhylogenies analyses we used the following criteria: (1) We examined trace files visually using the program Tracer v1.3 (Rambaut and Drummond, 2003) to confirm a plateau in log-likelihoods had been reached; (2) we compared multiple independent MCMC runs by (a) checking to see if the same consensus topology was produced by the post-burn-in samples, (b) comparing the estimates of the posterior probabilities for each branch, and (c) comparing the harmonic means of the log-likelihoods and various parameter values (Table 3); (3) we also made an effort to ensure that sufficient samples from the MCMC chain were independent by examining the effective sample size (ESS) for values of interest using Tracer v1.3.

The ESS is calculated by dividing the post-burn-in chain length by an estimate of the auto-correlation time (ACT), which itself indicates how far apart two samples must be for their correlation to drop to zero, indicating they are independent. Each parameter has its own auto-correlation time. In our runs we found the ACT for the log-likelihood was usually between 5000 and 12,000. This indicates our MCMC sampling strategy (once every 1000 steps) was too frequent thereby yielding overly large file sizes and inflated precision (which happens when the number of independent samples is lower than the total number of samples). Ideally, one should obtain large (>200) effective sample sizes for values of interest although an ESS of 100 is considered adequate (Rambaut and Drummond, 2003; A. Rambaut, in litt., April 2006). Effective sample size values below 100 indicate the estimate of the posterior distribution of that parameter is poor and the chain should be run longer to obtain more independent samples or one can combine independent, converged runs together to increase the ESS.
2.7. Maximum-likelihood bootstrapping

We obtained maximum likelihood estimates of the parameters of the GTR + I + G model using PAUP* 4.0b10 with the combined dataset from a neighbor joining tree. These estimates were: relative base frequencies $pA = 0.33306$, $pC = 0.13840$, $pG = 0.12351$, $pT = 0.40503$; substitution rate matrix values: $A-C$ 1.6958, $A-G$ 19.7072, $A-T$ 5.8133, $C-G$ 0.3940, $C-T$ 40.9085; shape parameter of the four-category discrete Gamma distribution, alpha = 0.8184; proportion of invariant sites = 0.6061. These parameter values were fixed for the duration of 500 bootstrap replicates each started from NJ trees with each replicate limited to 1000 trees examined via TBR branch swapping.

2.8. Morphometrics

We conducted two sets of morphometric analyses on log-transformed traits to test the validity of 'N. praedator'. First, we used allometric analyses to test whether 'N. praedator' individuals have a less transverse pronotum than N. investigator. Lacking reliable morphological diagnostic characters to identify 'N. praedator' specimens we used instead a combination of geography and morphology to sort specimens into two categories: N. investigator and potential 'N. praedator'. Pronotum greatest width, least width, and pronotum length were measured on 91 specimens of N. interruptus, 210 specimens of N. investigator and 129 specimens of potential 'N. praedator' (Table 4). Observations were made with a Wild M3C stereo dissecting microscope (80× max; Heerbrugg, Switzerland) and fiber optic lights. Measurements were made using an ocular micrometer or using digital calipers (VWR, repeatability: 0.01 mm). Geometric mean regressions were performed using the Model II program (Legendre, 2001). Geometric mean regressions may be more appropriate than least-squares regressions when variables on both the $x$- and $y$-axes are measured with error (Sokal and Rohlf, 1995), which applies when considering the relationship between two morphometric traits. We performed separate regression analyses of log10 (hereafter, log) pronotum "transverse ratio" (PTR; i.e., log pronotum greatest width/log pronotum least width) on log pronotum length for males and females (e.g. Butler et al., 2000; Colgoni and Vamosi, 2006; Tomkins et al., 2005). When examining outputs from the Model II program, it was noted that major axis (MA) and ordinary least-squares (OLS) regression analyses produced comparable slope and intercept estimates for all species × sex combinations (e.g. male N. investigator: MA slope = −0.033, MA intercept = 1.072; OLS slope = −0.029, OLS intercept = 1.069). Thus, subsequent analyses of differences among groups (e.g. heterospecific males) were conducted using general linear models with JMP 5.1 (SAS Institute, Cary, NC, USA), rather than simply comparing 95% confidence limits of these estimates (see also Colgoni and Vamosi, 2006). For these analyses, we are particularly interested in examining equality of slopes (i.e., a significant interaction between group membership and log pronotum length on PTR).

Second, we complemented the allometric analyses with separate discriminant function analyses for males and females in the three species. These analyses were conducted with a subset of our samples (48 males, 56 females) for which we had additional two additional body size measures: elytron length (measured from the humerus to the posterior edge) and elytron width at the humeri. The validity of 'N. praedator' would be supported if potential 'N. praedator' individuals were typically distinguishable from N. investigator based on these measurements.

2.9. Biogeography—ancestral character state reconstruction

Mesquite v1.1 (Maddison and Maddison, 2005b) and MacClade v4.04 (Maddison and Maddison, 2005a) were used with the combined analysis Bayesian phylogram to infer the geographic distribution of ancestral taxa. For all reconstructions we first pruned the taxa to single samples per species because multiple samples per species could influence the reconstruction probabilities for ancestral nodes—except for the Holarctic species N. investigator for which we left in one Old World and one New World terminal. Parsimony and the Lewis (2001) Mkv symmetric 1-parameter model, in addition to the asymmetrical Mkv 2-parameter model (which allows forward and backward rates to differ) were used to map a binary distribution character (Old World/New World). Rather than rely only on the consensus (point-estimate) phylogeny we also estimated ancestral states using all 6000 post-burn-in trees from the first two 3Q GTR + G runs. This was done in the manner described by Lewis and Lewis (2005), which allows credible intervals to be constructed for each ancestral state hypothesis. The trees sampled from the MCMC chains were brought into PAUP* 4.0b10 (Swofford, 2001) and the number of transitions between Old World and New World, under both ACCTRAN and DELTRAN parsimony was counted for all trees. This was done for both a full-OTU dataset and a trimmed dataset with only one terminal per species (except N. investigator, see above).

We also used SIMMAP 1.0b2.1 (Bollback, 2006) to estimate the number of state changes between Old World and New World across the 3000 post burn-in trees from our first 3Q GTR + G MCMC analysis. We used an asymmetrical 2-parameter Markov model for these estimates although it is not clear whether inferred molecular branch lengths should be allowed to influence the estimated probabilities of biogeographic state changes ("Are taxa with long molecular branches more likely to experience biogeographic/distributional changes than taxa with short or average branch lengths?" Towards this end we compared these results with those obtained with branch lengths estimated under a molecular clock).

2.10. Genetic distances

To determine if within and among species genetic distance patterns corresponded to species boundaries we used PAUP* 4.0b10 to examine both corrected (GTR + G) and uncorrected ‘p’ distances. Uncorrected distances are shown, acknowledging that at least the larger distances will be significant underestimates of actual distances—however, our interest is primarily in the smallest distances.

2.11. Hypothesis testing

We used the following Bayesian method to test specific hypotheses of species monophyly (exclusivity): Post-burn-in trees sampled from BMCMC runs were imported into SIMMAP 1.0b2.1 and filtered to retain trees compatible with a constraint topology built.
to enforce the hypothesis in question. The proportion of trees compatible with the constraint is the estimated posterior probability of that hypothesis (Carstens et al., 2004; Lewis and Lewis, 2005).

3. Results

3.1. Sequences

Sequences of the mitochondrial genes COI and COII were obtained for 50 individuals representing 16 species of the genus Nicrophorus (Appendix A). Eight of these samples represented outgroups and are members of the vespillioides and marginatus species-groups (sensu Sikes, 2003). The 42 ingroup samples were all members of the investigator species-group. These sequences are available from Genbank (EF537596–EF537645) and the aligned NEXUS file is available from TreeBase (http://www.treebase.org) under study Accession number S2042.

The final alignment, including outgroups, was 2129-bp-long and was checked against its published translation to confirm the alignment (no frameshifts or stop-codons, etc.). Our COI sequences for these Nicrophorus contain 1304 bp, but are incomplete—starting at site 225 of the sequences published by Lunt et al. (1996), which is a second codon position, and terminating at their site 1528. Our COI sequences are complete—starting at site 1 (first codon position) of the Liu and Bekenbach (1992) sequences and terminating with a single T of the stop codon at their site 688. Of these 2129 bp, 1517 are constant and 45 are variable but parsimony-uninformative, leaving 567 parsimony-informative sites (413 ingroup informative). COI has 360 informative sites of 1304 (28%)—within the ingroup (investigator species group) COI has 269 informative sites (21%), COII (plus the tRNAs Leu, Asp, and Lys) has 207 informative sites of 825 (25%)—within the ingroup it has 144 informative sites (18%). Among the 664 codons in the coding sequences 81 (12%) of the first position sites, 12 (1.8%) of the second position sites, and 456 (69%) of the third position sites were parsimony informative. Of the 137 non-coding sites only 17 (12%) were parsimony informative.

Insect mitochondrial DNA is typically A–T-rich (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%). The complete mtDNA dataset included 31.9% A, 15.3% C, 14.2% G, and 38.6% T, values which are typical for insect mtDNA (Frati et al., 1997). Our dataset showed this strong A–T bias (71%).

Table 5 shows the rate parameter estimates for each rate matrix from two 5-million step MCMC runs of the combined data using BayesPhylogenies. The relatively similar weights of the three separate rate matrices indicate that each is explaining a substantial portion of the data. A parameter for invariant sites cannot be set low a rate matrix to identify and fit such sites, if they exist. The rate estimates for the combined data indicate none of the matrices conform with an invariant sites model—all show rates well above zero. Additionally, all matrices agree with a high number of transition events.

Three 3-rate matrix GTR + G MC3 runs using the program BayesPhylogenies resulted in the same 50% consensus topologies which agreed more with the COI partition ML tree (Fig. 4A) than the COII partition ML tree. However, given the stronger fit of the a priori partitioned GTR + G model under MrBayes, the final topology (Figs. 5 and 6) that we chose to represent our best estimate of the phylogeny is a 50% majority rule consensus phylogram of 1000 post burn-in trees sampled from two separate 2-chain MCMC runs using MrBayes. The BayesPhylogenies analyses all appeared to converge to the same probability space as evidenced by the posterior probabilities of branches from independent analyses being highly correlated ($R^2 = 0.9981, P < 0.001$).

Peck and Anderson’s (1985) morphology-based cladistic work represents six hypotheses of relationships among the investigator and marginatus group species (Fig. 1): (tomentosus, hybridus),

<table>
<thead>
<tr>
<th>OTU</th>
<th>Observed/expected</th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>semenowi2</td>
<td>O</td>
<td>127</td>
<td>28</td>
<td>9</td>
<td>147</td>
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<tr>
<td>E</td>
<td>129.08</td>
<td>30.88</td>
<td>10.72</td>
<td>140.32</td>
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</tr>
<tr>
<td>sepulcor1</td>
<td>O</td>
<td>134</td>
<td>37</td>
<td>10</td>
<td>130</td>
</tr>
<tr>
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<td>30.88</td>
<td>10.72</td>
<td>140.32</td>
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</tr>
<tr>
<td>marginatus1</td>
<td>O</td>
<td>126</td>
<td>65</td>
<td>22</td>
<td>98</td>
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<td>E</td>
<td>129.08</td>
<td>30.88</td>
<td>10.72</td>
<td>140.32</td>
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</tbody>
</table>
Fig. 4. Ninety percent majority rule consensus trees for separate 3Q MC³ GTR + G analyses using BayesPhylogenies of each gene. (COI) Based on 1304 bp analysis of COI mtDNA sequence data. (COII) Based on 825 bp analysis of COII mtDNA sequence data. There are only three well-supported (>90% PP) contradictory branches between these trees (marked with lower case Roman numerals).

The remaining lineages of the investigator group are less well separated and supported. The most important branches with low support are those in the mid-level of the tree and involve the species Nicrophorus semenowi, Nicrophorus interruptus, and (N. mexicanus + N. nigrita). Each of these species is monophyletic (exclusive) with strong support although we only had one sample of the rare species N. semenowi and the four samples of N. nigrita are monophyletic at the slightly low MLboot = 75% but fairly high PP = 0.94. Our suspicion, based on their close genetic distances, that N. mexicanus and N. nigrita would fail reciprocal monophyly was incorrect.

The Old World species N. semenowi was problematic. It either joined to the New World lineage as sister to the pair N. nigrita

<table>
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<tr>
<th>Tested topology</th>
<th>Tested dataset</th>
<th>COI</th>
<th>COII</th>
<th>All data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COI</td>
<td>COII</td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>COI</td>
<td>Best (best)</td>
<td>0.000 (0.000)</td>
<td>0.262 (0.244)</td>
<td></td>
</tr>
<tr>
<td>COII</td>
<td>0.000 (0.000)</td>
<td>Best (best)</td>
<td>0.028 (0.015)</td>
<td></td>
</tr>
<tr>
<td>All data</td>
<td>0.134 (0.347)</td>
<td>0.047 (0.121)</td>
<td>Best (best)</td>
<td></td>
</tr>
</tbody>
</table>

In parenthesis are given the P values for tests done with three problematic OTUs removed (see text for details).

Table 6

<table>
<thead>
<tr>
<th>Rate matrix</th>
<th>Rate parameters</th>
</tr>
</thead>
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<tr>
<td>A&lt;-&gt;C</td>
<td>A&lt;-&gt;G</td>
</tr>
<tr>
<td>C&lt;-&gt;G</td>
<td>C&lt;-&gt;T</td>
</tr>
<tr>
<td>Q1</td>
<td>2.646</td>
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<tr>
<td>Q2</td>
<td>1.852</td>
</tr>
<tr>
<td>Q3</td>
<td>5.127</td>
</tr>
</tbody>
</table>

Values are means from the first two 5-million step MCMC runs. Transitions are in bold. Matrix weights (Q_{weight}) sum to 1. The weight of a matrix is proportional to the amount of the data it explains.

Table 7

They acknowledged the possibility that some Old World taxa might fall within their species groups and so we held their hypotheses to a test of paraphyly rather than monophyly. All of Peck and Anderson’s (1985) hypotheses of relationships were strongly supported by our results (PP > 0.90). All branches of the marginatus group and the basal lineage within the investigator group, Nicrophorus tomentosus and Nicrophorus hybridus, including the monophyly of these respective species groups, were well supported at PP = 1.0 and MLboot (maximum likelihood bootstrap) >90%. Species in this portion of the phylogeny were monophyletic and separated from their nearest relatives by relatively long branches.
and *N. mexicanus* or joined to the Old World lineage as sister to the crown clade of *N. interruptus* through *N. investigator* resulting in a very short branch that collapsed in bootstrap and most Bayesian analyses but was recovered as sister to the New World clade at PP = 0.71 in the MrBayes analysis (Fig. 5). Ignoring the Holarctic species *N. investigator* and *N. vespilloides*, the former placement requires two geographic steps and has two equally parsimonious reconstructions, whereas the latter placement requires only one geographic step and has only one parsimonious reconstruction.

There are two first-codon position and nine third-codon position changes that would be synapomorphic for the Old World polyphly placement (four of these 11 changes are transversions) whereas there are three first-codon position and six third-codon position changes that would be synapomorphic for the Old World monophyly placement (three of these nine changes are transversions). There were no second-codon position changes involved in either hypothesis. None of these changes, for either hypothesis, have a consistency index of 1.0 (all are homoplastic to some degree). Of these five first-codon changes for each hypothesis, four are synonymous. The nonsynonymous change would be a synapomorphy for the New World placement (Old World polyphly) and involves a change between valine and isoleucine as the final amino acid of the COII sequence. Most of the OTUs have isoleucine at this site but valine is seen in *N. semenowi* and the New World species *N. nigrita* and *N. mexicanus* and the New World outgroup species *N. obscurus* and *N. guttula*. Morphologically, *N. semenowi* shares with *N. nigrita* and *N. mexicanus* the rare state of dark brown metasternal pubescence (Sikes, 2003).

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**Fig. 5.** Inferred phylogeny of *Nicrophorus investigator* and *marginatus* (in part) species groups. 70% Majority rule consensus phylogram of 2002 post burn-in trees from two independent 2 million MCMCMC (2 chains) runs, sampled once every 1000 steps, yielding 2000 trees per run using a three partition GTR + G model with the software MrBayes. Maximum-Likelihood bootstrap values (500 replicates, 1000 TBR swaps each, GTR + I + G model) and estimates of Bayesian Posterior Probabilities are provided for each branch. See Fig. 6 for detail on the crown clade of *N. investigator* and *N. encastus*.
Fig. 6. Inferred phylogeny of the Holarctic species Nicrophorus investigator and the Himalayan endemic N. encaustus (detail on the crown clade of Fig. 5; see caption of Fig. 5 and Section 2 for details of analysis). ML bootstrap values are absent for branches that were not present in the 50% majority rule consensus bootstrap tree. Samples from regions of potential ‘N. praedator’ are those from Russia and Japan.

The two most derived lineages {N. argutor + (N. sepultor + N. confusus)} and {N. investigator + N. encaustus} are sisters at PP = 1.0; ML boot = 74% and each lineage is supported at PP = 1.0; ML boot = 100%. The questionable species N. confusus was found to be paraphyletic (nonexclusive) with respect to N. sepultor—whereas the clade (N. confusus + N. sepultor) was well separated from, and monophyletic with respect to, N. argutor (both PP = 1.0; ML boot = 100%). The post burn-in BayesPhylogenies MCMC trees were filtered to determine what percent agrees with monophyly of N. confusus. This method rejected N. confusus monophyly at PP = 0.17. All 21 samples of N. investigator, including those from regions of potential ‘N. praedator’, held together in a clade with PP = 1.0; ML boot = 100%. However, N. encaustus joined within the basal samples of N. investigator, making this latter species paraphyletic (nonexclusive). We filtered the post burn-in MCMC trees to determine the posterior probability of N. investigator monophyly which was strongly rejected at PP = 0.0000. Given the topology of Fig. 5 we were not surprised that the hypothesis of Khatchikov and Popov (2006), that N. interruptus is a subspecies of N. investigator, was also strongly rejected at PP = 0.0000.

Within-species phylogenetic structure was seen with individual, well-supported clades of N. investigator samples from the New World (PP = 1.0; ML boot = 90%), Siberia (PP = 1.0; ML boot = 84%), and Hokkaido respectively (PP = 0.97; ML boot = 77%) (Fig. 6). Three samples from China and two from Honshu Japan were the only non-monophyletic geographic region sampled, with one Chinese OTU in part of a basal polytomy, one as sister to N. encaustus, and one as sister to the Siberian samples. One Honshu sample formed a weakly supported sister clade to the four samples taken from North America, (PP = 0.65; well supported PP = 1.0 under BayesPhylogenies, Fig. 7). However, the Bayesian results disagreed with the maximum likelihood bootstrap analysis that found the Japanese samples to be weakly monophyletic at ML boot = 71% (tree not shown). We did not detect evidence of a cryptic species corresponding to N. praedator in our results (Fig. 6).

3.4. Genetic distances

Within and among taxa genetic distances show large overlaps between different comparisons (Fig. 8). Contrary to expectation, the outgroup, N. vespilloides, shows only the second greatest distances (11.0%) from the ingroup samples (Fig. 8A). The greatest mean and maximum distances are seen in the comparisons between the marginatus group and the investigator group species (Fig. 8Aii). Although the marginatus group is phylogenetically closer to the investigator group than the vespilloides group, its distances completely overlap those of the vespilloides group.

When all species are included, the among-species comparisons within the investigator group average 7.04% but show a minimum of 0.33% (Fig. 8A–iii and B). When the most closely related species are removed, some of which are of questionable validity due to a lack of diagnostic characters, the minimum distance between species raises to 2.89%—still quite low (Fig. 8A–iv). This suggests that some of the species in this group have not diverged much since speciation. When comparisons are limited to just the most closely related species, the distances range from 0.33% to 3.0% (Fig. 8A–v and B). These closely related species pairs are {N. encaustus vs. N. investigator}, {N. confusus vs. N. sepultor}, and {N. mexicanus vs. N. nigrita}.

Within species comparisons (Fig. 8A–vi) overlap completely the among-species comparisons of the most closely related species (Fig. 8A–v). In fact, the maximum within-species distance, 3.76%, was far greater than the minimum among-species distance, 0.33%. Earlier work using COI alone found a gap of almost 3% that separated the lowest among-species comparison (5%) with the largest within-species comparison (2%) for the entire subfamily (Sikes et al., 2002; Sikes, 2003). Because of the very small distances between these closely related species, no such gap was found in this current dataset—making it impossible to infer species status from genetic distances alone (i.e. there is no genetic distance “cut-off” below which a comparison is always conspecific, that is, unless we changed the taxonomy by synonymizing all these closely related species with their respective sister species).

Restricting our focus to just the closely related species pairs shows that the within-species distance of N. confusus (0.471%) is very close to the between-species distance comparing N. confusus to N. sepultor (0.518% and 0.329%)—this agrees with the paraphyly seen for N. confusus in Fig. 5—one N. confusus sample is closer to the N. sepultor sample than it is to the other N. confusus sample. This is also true for N. encaustus and N. investigator. The two N. encaustus samples are 0.0% distant from each other, and their distance to N. investigator is much greater (1.76–3.01%)—although there are samples of N. investigator which are closer genetically to N. encaustus than they are to other samples of N. investigator, accounting for the paraphyly. Paraphyly is not seen in Nicrophorus mexicanus which shows greater between species distances (0.71–1.13%) to its nearest relative, N. nigrita, than either species shows within: N. mexicanus (0.19%), N. nigrita (0.14–0.42%).

3.5. Morphometric results

Patterns of allometry were most similar between N. investigator and potential ‘N. praedator’ (Fig. 9), although some differences were found in the degree of sexual dimorphism in the ratio of log pronotum/tarsus:log pronotum/tarsus least width (i.e., PTR). We detected a significant interaction between sex and log pronotum
Fig. 7. Scatterplot of estimated posterior probabilities of BayesPhylogenies 3Q GTR + G analyses with the combined dataset versus estimated posterior probabilities of MrBayes three partition GTR + G analysis.

Fig. 8. Among and within species uncorrected ‘p’ genetic distances for combined COI and COII dataset including adjacent tRNAs. (A) Min–max comparisons (i) among species, outgroup vs ingroup—vespilloides species group vs investigator species group, mean = 11.66%, n = 252; (ii) among species, investigator species group vs investigator species group, mean = 11.00%, n = 84; (iii) among species, investigator species group, all species, mean = 7.04%, n = 636; (iv) among species, investigator species group, minus distances between the three most closely related, questionable species (N. encaustus/N. investigator, N. nigrita/N. mexicanus, N. sepultor/N. confusus), mean = 8.19%, n = 97; (v) among species, investigator species group, restricted to the three most closely related, questionable species and their sister species, mean = 2.29%, n = 52; (vi) within species of the investigator species group, mean = 1.77%, n = 225 (note that if N. praedator is among our samples its distance to N. investigator would be within this last group). (B) Histogram of distance comparisons, black bars: among species contrasts; white bars: within species contrasts (note large number of among species contrasts <4%).

length ($F(1,206) = 4.83$, $P = 0.029$) in N. investigator. Large males had considerably higher PTR values than large females in this species (Fig. 9). In potential ‘N. praedator’, there was a marked difference between the sexes in PTR values ($F(1,125) = 39.20$, $P < 0.0001$). However, there was neither an interaction between sex and log pronotum length ($F(1,125) = 1.61, P = 0.21$) nor a main effect of log pronotum length ($F(1,125) = 0.79, P = 0.38$) on PTR. Finally, in N. interruptus, there was again no evidence for an interaction between sex and log pronotum length ($F(1,86) = 0.23$, $P = 0.63$). Although there was again a difference between the sexes ($F(1,86) = 8.01, P = 0.006$), the strongest pattern was a main effect of log pronotum length ($F(1,86) = 1.26, P = 0.26$) on PTR with large individuals typically having a less transverse pronotum than smaller individuals (Fig. 9).

In comparisons of heterospecific males, we found no differences between N. investigator and potential ‘N. praedator’ males for either main effect or their interaction (all $P > 0.47$). Conversely, there was evidence for a marginally significant interaction between species and log pronotum length ($F(1,98) = 3.10, P = 0.08$) when comparing N. interruptus and potential ‘N. praedator’ males. Comparisons between males of N. investigator and N. interruptus again highlighted potential differences between these species, with a marginally significant main effect of species on PTR ($F(1,98) = 2.95, P = 0.088$). Thus, potential ‘N. praedator’ and N. investigator males are the most similar to one another according to these allometric analyses.

Discriminant function analyses revealed considerable overlap between particular groups, with the proportion of males (16.7%) that was misclassified being lower than that for females (33.9%). Potential ‘N. praedator’ individuals tended to have intermediate scores on the first, and lower scores on the second, discriminant axis than N. investigator and N. interruptus (Fig. 10). In males, all N. investigator individuals were correctly classified, whereas 14.3% of N. interruptus males were misclassified as potential ‘N. praedator’. Although most potential ‘N. praedator’ males were correctly classified, misclassifications happened more frequently to N. investigator (18.8%) than to N. interruptus (6.3%). It is worth noting that in this analysis no male N. investigator were misclassified as N. interruptus, and the converse was also true. In females, most misclassifications were between N. investigator and potential ‘N. praedator’, as expected from the allometric analyses, to such an extent (33%) that females of these two groups cannot be considered to be distinguishable based on these characters. Similar to the pattern observed in males, only a single (6.7%) N. investigator was mis-
species group is also restricted to the New World. Because the outgroup sample of the Holarctic species \( N. \) vespilloloides is Old World, the most parsimonious reconstruction of the ancestral distribution (Fig. 11a) is equivocal about the ancestral state for nodes A, B, and C in Fig. 11a.

The asymmetric, two-parameter Markov model reconstructs the common ancestor of the species group (node B, Fig. 11b) as the state ‘Old World’ with a 56% probability but this value does not pass the ‘decision threshold’ so this reconstruction is not significantly decisive. The next node (node C, Fig. 11b) is equivocal under parsimony but weakly (76%) reconstructed as Old World by the Markov model. Branch support for this node varied (PP = 0.4–0.95%) depending on the complexity of the model and partitioning approach used (Fig. 12). Requiring branch lengths to enforce a molecular clock, and thus better represent time, did not change these conclusions (node A, New World 67%; node B, New World 51%; node C, Old World 82%).

In summary, parsimony provides no answer for the ancestral distribution using this point estimate approach whereas the likelihood method provides weak, but not decisive, support for these ancestors being Old World although derived from an ancient New World species at 54% probability (node A, Fig. 11B). This approach supports, albeit weakly, an initial transition to the New World which gave rise to the Nearctic marginatus group species, and an ancestor that returned to the Old World from which were later derived the New World lineages (\( N. \) tomentosus, \( N. \) hybridus) and (\( N. \) nigrita and \( N. \) mexicanus) and the remaining Old World species of the investigator group.

Fig. 9. Allometric relationships between log pronotum length and PTR (i.e., log pronotum greatest width/log pronotum least width) for male (solid symbols and line) and female (open symbols and dashed lines) \( N. \) interruptus (top), \( N. \) investigator (middle), and potential ‘\( N. \) praedator’ (bottom). Lines shown are slopes for significant major axis regression analyses.

Fig. 10. Results of discriminant function analyses on male (top) and female (bottom) \( N. \) interruptus (■, males; □, females), \( N. \) investigator (●, males; ○, females), and potential ‘\( N. \) praedator’ (▲, males; ◆, females).

classified as \( N. \) interruptus, and a single (5%) \( N. \) interruptus was misclassified as \( N. \) investigator.

3.6. Biogeography

The basal-most lineage of the investigator species group, comprising the sister species \( N. \) tomentosus and \( N. \) hybridus, is restricted to the New World (Fig. 11). The immediate outgroup of the investigator species group, comprising three members of the marginatus
Basing ancestral state inferences on a point estimate of phylogeny, even a consensus of MCMC trees, can be misleading and fail to accommodate the variation in the phylogenetic signal. We therefore also investigated this question using more general Bayesian methods. Under parsimony, based on the uncertainty contained in the 6000 post-burn-in trees of the first two 3Q GTR + G MCMC runs, a reconstruction of four transitions between Old and New world had the highest posterior probability (0.8227) with the remaining probability contained in a hypothesis of three transitions (0.1773). An ACCTRAN reconstruction (which favors reversals) of the four-change hypothesis counted three changes from Old World to New with a single reversal, whereas under DELTRAN (which favors parallelisms) all four state changes were from Old World to New. The ACCTRAN reconstruction implies the sampled Old World species of the investigator group descended from a New World ancestor. The DELTRAN reconstruction, on the other hand, favored no re-invasion of the Old World from New World stock and explained all current distributions as movement from Old World to New. This hypothesis fits that of Peck and Anderson (1985) who seemed to prefer a scenario in which all dispersal was from Old to New world with no reversals. Unfortunately, parsimony offers no objective means by which one can decide which of these two optimizations is the more probable, so we used a more fully Bayesian approach.

Using the program SIMMAP 1.0b2.1 we accommodated uncertainty in ancestral state estimates by counting across 3000 post-burn-in trees from the first 3Q GTR + G MCMC run. Unlike parsimony which divided all the posterior probability between two hypotheses (three changes versus four), the two-parameter Markov model found 10 hypotheses with non-zero probability. These were (number of changes: posterior probability): 3, 0.0289; 4, 0.7161; 5, 0.1092; 6, 0.1102; 7, 0.0206; 8, 0.0103; 9, 0.0026; 10, 0.0006; 11, 0.0003; and 14, 0.0003. However, in agreement with the parsimony analysis the four-change hypothesis captured the greatest posterior probability (0.7161).

Within the four-change hypothesis the Markov model identified 575 (27%) of the trees that were consistent with four changes from Old World to New, 1540 (72%) of the trees that were consistent with three changes from Old World to New with one reversal, and 35 (1.6%) of the trees that were consistent with two changes in both directions. Thus, the Markov model applied across 3000 post-burn-in trees placed the most weight on the four change hypothesis that agreed with the ACCTRAN parsimony optimization—altogether this hypothesis received the greatest posterior probability. Contrary to expectations (e.g. Peck and Anderson, 1985) these results indicate the sampled Old World species of the investigator group probably descended from New World stock (i.e. the ancestors at nodes A and B in Fig. 11 were probably New World species).

4. Discussion

4.1. Phylogenetic results

Although the ILD and SH tests indicated there was significantly different signals between the two gene partitions, the 90% majority
rule Bayesian topologies from each gene (Fig. 4) are very similar—
differing only in weakly supported branches, with three exceptions:
two Chinese samples and one Japanese (Honshu) sample of N. investigator. When these three OTUs are removed, the null of
homogeneity is not rejected. Although there are good arguments to
not combine significantly heterogeneous data (Bull et al., 1993)
there are also good arguments to combine (Adkins et al., 2001;
Flynn and Nedbal, 1998; Pereira et al., 2002; Sullivan, 1996). We
have done so here because the few strongly supported contradic-
tions between these different gene trees do not bear on the tests of
species status that are the main focus of this study. The differ-
ences are limited to branches inside the widespread and Holarctic
species N. investigator and both genes agree that N. encaustus falls
inside this clade.

4.2. Inflated branch support

There are two weakly supported branches in the mid-level of
the tree. The better supported of the two is the branch uniting
the species N. interruptus with the more derived “crown” clade of
N. investigator + (N. sepultur, N. confusus, and N. argutor). The weak-
er branch is that holding N. semenowi to the species pair N. mexic-
amus and N. nigrita. We noticed both of these branches received
higher support when simpler models (GTR, 1Q GTR + G) were used
and lower support with more complex models (Fig. 12).

The high support with simple models could be an artifact due to
model under-fitting (Sullivan et al., 1997; Erixon et al., 2003; Huel-
lsenbeck and Rannala, 2004; Lemmon and Moriarty, 2004) or an
inherent bias in current Bayesian methods dealing with short
internal nodes (Lewis et al., 2005). Alternatively, the low support
with more complex models may be an artifact due to excessive
variance resulting from model over-fitting.

Buckley and Cunningham (2002) attempted to identify cases of
artifacts resulting from model over-fitting using nonparametric
bootstrapping and complex likelihood models used to analyze
empirical datasets. They confidently identified artifacts due to un-
der-fitting, but no case of over-fitting was identified. However,
they were using maximum-likelihood bootstrapping which has
been shown to be more robust to model violation than Bayesian
inference (Huelsenbeck and Rannala, 2004). Even with Bayesian
methods, which allow vastly greater model complexity due to
the ability to use meta-models composed of multiple sub-models,
there has been only minor evidence of slightly lower support
resulting from use of a too-complex model (eg. Huelsenbeck and
Rannala, 2004; Nylander et al., 2004; Lemmon and Moriarty,
2004). With extremely complex models, (e.g. 12 partitions and
121 free parameters not including branch lengths, Nylander
et al., 2004) issues of convergence and adequate mixing in the
MCMC run, both of which seem harder to achieve, have been iden-
tified as requiring more study. Huelsenbeck and Rannala (2004)
were unable to detect any negative consequences of over-fitting
when data were generated under a model significantly simpler
than the model used to analyze (e.g. JC69 + GTR + I + G). There
has, however, been ample demonstration that under-fitting the
data in a Bayesian analysis can lead to artifactually high branch
support (Huelsenbeck and Rannala, 2004; Lemmon and Moriarty,
2004), therefore we interpret these results as indicating the sup-
port under the simpler models to be exaggerated and the lower
support under the chosen model (Figs. 5 and 12) to be closer to
accurate.

Related to this issue of model complexity and interpretation of
varying support values in a Bayesian context is the identification of
an artifact in Bayesian analyses related specifically to short internal
branches and polytomies (Lewis et al., 2005). Lewis et al. (2005)
explained how current implementation of Bayesian MCMC can re-
sult in unusually high support for short internodes relative to sup-
port obtained from ML-bootstrapping. Their work possibly
explains a number of anomalous results in the literature (dubbed
“The Bayesian Star Tree Paradox”) in which Bayesian methods re-
turned strong branch support for branches that should have been
collapsed into polytomies (e.g. Suzuki et al., 2002). Specifically,
their findings indicate that these artifactually high branch support
values can be obtained without model violation. The solution they
propose has yet to be incorporated into Bayesian phylogenetic soft-
ware, so, until it is incorporated, there is an extra reason to com-
pare Bayesian branch supports to Maximum Likelihood Bootstrap
values. In this case (Fig. 12) ML bootstrapping returned a low value
of 32% for the N. semenowi clade whereas the less complex, 1 pat-
tern, Bayesian implementations of the GTR model returned high
support (0.97 PP)—further evidence these high values are mislead-
ing. The same was true for the N. interruptus branch—ML boot-
strapping returned a low value of 59% while the 1-pattern GTR
model returned high support (1.0 PP). In both cases, use of the
more complex 3-pattern GTR + G model returned low support for
these branches in agreement with ML bootstrap results (Fig. 12).
This is an important finding bearing on the problem of model
underfitting in Bayesian analyses.

4.3. BayesPhylogenies vs. MrBayes

Although Pagel and Meade (2004, 2005) provided theory and
results (both empirical and simulation) indicating their mixture
model approach should yield a better fit to common molecular
datasets, we found an a priori partitioned MrBayes analysis fit
our data significantly better. To make the comparison as fair as
possible we used the same base model (GTR + G) and unlinked only
the rate matrices in both. Surprisingly, a 21-parameter a priori
three-partitioned analysis had a significantly better fit than a
four-pattern, 30-parameter, BayesPhylogenies analysis (Table 3).
The mean tree length of the MrBayes analyses was almost twice
that of the BayesPhylogenies analyses. The only important differ-
ence in the topology was the somewhat stronger support for the
N. semenowi + (N. nigrita and N. mexicanus) clade under MrBayes

Fig. 12. “Will the correct branch support please stand up?” Effect of model
complexity on estimated posterior probability of two mid-tree branches showing a
general decline in support with increased model complexity. White bars: N.
semenowi, typically this species joins to the pair of species N. nigrita and N.
mexicanus, although with weak support (32% MLBoot). Black bars: N. interruptus,
this Old World species typically joins to the base of the primarily Old World crown
clade of N. argutor, N. sepultur and N. investigator/N. encaustus, although with weak
support (59% MLBoot).
which was retained at PP = 0.71 but collapsed into a polytomy (PP < 0.60) in the BayesPhylogenies analyses (Fig. 12). Other differences in the trees included a number of the branches in the N. investigator clade (Fig. 6) that had much stronger support under BayesPhylogenies than MrBayes (Fig. 7). The lower values from the MrBayes analysis were much closer to the MLBoot values. It was not generally the case that support was higher with BayesPhylogenies—some branches showed the opposite pattern (Fig. 7). Pagel and Meade (2004, 2005) made a compelling case for their approach, which we’ve described in part above, and we hope further research explores their ideas more thoroughly.

4.4. Biogeography

Although the argument above suggests low support for these two branches is the most accurate representation of the signal in the data, biogeography can help us evaluate the credibility of these branches independently of the DNA results. The current placement of the Old World species N. interruptus in Fig. 5, although weakly supported, agrees with a monophyletic Old World crown clade and is thus biogeographically parsimonious. Using the same logic, the placement of the Old World species N. semenowi as sister to the Nearctic species pair N. nigrita and N. mexicanus makes the Old World crown clade paraphyletic (or polyphyletic) and is thus not biogeographically parsimonious. Some of the biogeographic homoplasy would vanish if N. semenowi joined at the base of the Old World crown clade, thus making the Old World species monophyletic (this would favor a 3-step reconstruction over the currently favored 4-step reconstruction to explain the biogeographic data). However, this placement of N. semenowi is seen in only 17% of the 3000 post-burn-in MCMC trees sampled from the first 3Q GTR + G analysis (in contrast to 58% which hold N. semenowi together with the Nearctic N. nigrita and N. mexicanus). Therefore, despite our conclusion that the low support for the placement of these two species is a more accurate depiction of the signal in the data, biogeography suggests the placement of N. interruptus is correct nonetheless, while that of N. semenowi is not.

There is one fundamental biogeographic question to answer for this group of species: Do any of the Old World species trace their ancestry on the issue of timing because there is no fossil evidence for N. interruptus of the Old World species together with the Nearctic N. semenowi and N. mexicanus. Therefore, despite our conclusion that the low support for the placement of these two species is a more accurate depiction of the signal in the data, biogeography suggests the placement of N. interruptus is correct nonetheless, while that of N. semenowi is not.

Peck and Anderson (1985) proposed possible timings of these invasions within the Tertiary or Pleistocene. They hypothesized that species found in more open and semi-arid habitats created by the rain shadow formed by uplift of the Rocky Mountains are younger and more derived, or represent later ancestral invasions. In contrast, we have found that one of the most grassland-associated Nicrophorus, N. hybridus, distributed in the prairies east of the Rockies (Fig. 2A), is actually a basal lineage within the species group. Although the lineage may be old, adaptation to its current ecological niche may have resulted from more recent evolutionary change, as suggested by Peck and Anderson (1985). We can say little on the issue of timing because there is no fossil evidence for these relatively young species nor do the data fit a molecular clock (results not shown) so we cannot assume a constant rate of change per unit time across the tree nor calibrate a tree for use with non-clock divergence dating methods.

4.5. Closely related species

One of the goals of this study was to examine a number of species of questionable validity due to their genetic closeness, lack of, or weak, morphological diagnostic traits, and issues of paraphyly (nonexclusivity) with their nearest relatives. Regarding these species we make the following comments in light of the criteria described by Wiens and Penkrot (2002).

4.5.1. Nicrophorus ‘praedator’ and N. investigator

The name Nicrophorus praedator has been used in 32 publications and treated as a valid species in all but the most recent—Sikes et al. (2002). These authors synonymized this name under N. investigator because this species name has a history of poor diagnoses and no reliable traits could be found to identify it. See Sikes et al. (2002) for a thorough justification and explanation of this problem. We have examined 338 specimens from within the supposed distribution of N. praedator (Japan, Korea, northeast China, eastern Russia [Ussuri]) and have been unable to sort specimens according to prior authors concepts of N. praedator/N. investigator. Despite, or perhaps due to, the lack of reliable characters for identification of N. praedator a number of Nicrophorus specimens have been misidentified as N. praedator. In 1964 Mroczkowski, a skilled coleopterist, misidentified a series of Nicrophorus basalis from Russia and Korea in the Hungarian National Collection (HNHM) as N. praedator. Other species that have been misidentified as N. praedator include specimens of N. chilenis (SMFD), N. nigricornis (SMFD), and N. japonicus (NHMW, FMNH, SMFD)—all of which are easily discernable from N. investigator. If N. praedator is not valid it is almost certainly a synonym of N. investigator, many specimens of which have also been misidentified as N. praedator in collections.

Although no authors prior to Sikes et al. (2002) have treated N. praedator as a synonym, many have reported N. investigator from regions of N. praedator, including many Japanese authors. If N. praedator does exist as a biological species it appears to be sympatric with, and less common than, N. investigator. Our phylogenetic results failed to discover any samples identified as N. investigator that were distinctly related to other N. investigator samples. The Russian and Honshu samples, in particular, which we expected to be the most likely to belong to N. praedator, showed very low genetic divergences from other N. investigator samples and clustered within them. This lack of distinction was corroborated with larger sample sizes in our morphometric analyses. Certainly, our discriminant function analyses failed to consistently distinguish potential ‘N. praedator’ from N. investigator, while being able to distinguish the two definitely valid species, N. investigator and N. interruptus, from each other. Although the allometric analyses failed to find strong contrasts among the outcomes of various heterospecific male comparisons, potential ‘N. praedator’ and N. investigator males were most similar to one another in the relationship between pronotum “transverseness” and body size. We therefore retain N. praedator as a junior synonym while admitting that it is challenging to reject a hypothesis of existence.

4.5.2. Nicrophorus encaustus and N. investigator

Considerable structure was found in the intraspecific phylogeny of the widespread and Holarctic species N. investigator. The larger genetic distances (e.g. 3.1%) within this species exceeded those seen between other species pairs, such as N. argutor and N. sepultror (2.9%). This result is not surprising given the widespread Holarctic distribution of N. investigator, and the larger number of distant population samples we obtained for this species.

The phylogeny indicates N. investigator is paraphyletic with respect to N. encaustus. Other authors, such as Wiens and Penkrot (2002), prefer the term ‘nonexclusive’. They provide a flow-chart protocol to help delimit species based on haplotype phylogenies. Our results appear to match their Fig. 1c or d most closely in which the focal species, N. investigator, is non-exclusive with respect to
one or more distinct, exclusive species. Either the focal species is in fact multiple species (their Fig. 1c)—a conclusion one would reach if there was no evidence of gene flow among the basal lineages of the focal species, or the focal species is a single, non-exclusive, species assuming evidence of gene flow among the basal lineages could be found. Given the polytomy among the basal lineages and the placement of the Chinese samples (Figs. 5 and 6) there appears to be insufficient evidence of monophony among the basal lineages within this species, therefore supporting their Fig. 1d—N. investigator is a single paraphyletic (non-exclusive) species. This assumes there is justification for retention of N. encaustus as a distinct, valid species, which we feel there is, based on its simple diagnosibility from N. investigator. This Himalayan endemic, N. encaustus, has black antennal clubs while the Holarctic N. investigator has orange antennal clubs.

As more within species phylogenetic investigations are performed, paraphyly of widespread species is being found to be more common than initially thought. Funk and Omland (2003) performed a literature review and found 26.5% of 702 arthropod species in 143 studies were not monophyletic. The causes of paraphyly in this, or any case, can be difficult to discern. We have ruled out an explanation of artifact due to misidentification or lab contamination. There remains the possibility that the gene tree we have inferred is not the species tree. These species are largely allopatric although a small region of potential overlap exists in the far western portion of the Himalayan range of N. encaustus. However, DNA samples were taken from the far eastern portion of this species’ range—in complete allopatry with N. investigator and thus introgressive hybridization is a less likely explanation (Funk and Omland, 2003). A more likely explanation is incomplete lineage sorting—which is most detectable and more likely to produce non-monophyletic species when speciation is still ‘in progress’ and species are young or incipient (Avise, 1989; Buckley et al., 2006; Funk and Omland, 2003). Incomplete lineage sorting is more commonly a problem with nuclear DNA than mitochondrial due to the smaller effective population size of the latter (Moore, 1995) but can, nonetheless, cause the pattern seen here.

The explanation we prefer, given the obvious phylogeographic structure and widespread nature of the ‘source’ species N. investigator is a hypothesis of peripheral isolation combined with incomplete lineage sorting in which the isolate, N. encaustus, achieved monophony while retaining the phylogenetic signal linking it to its source population within the much more widespread N. investigator. Funk and Omland (2003) state “…peripheral isolates speciation may commonly yield a geographically restricted daughter species whose monophyletic set of haplotypes is embedded within a widely distributed and still paraphyletic parental species” and “In the case of budding speciation, forcing taxonomy to reflect gene tree monophyly by synonymizing the nested and parent species or by elevating lineages in the paraphyletic lineage to species status ignores the distinctive nature of the nested lineage” which supports our argument to retain both of these names as valid species.

4.5.3. Nicrophorus confusus and N. sepultor

The name N. confusus has appeared as valid in 16 of 17 publications (Sikes et al., 2002), all of which were taxonomic in scope (checklist, catalog, distributional records, keys, etc.). Kozminykh (1993) was the only author to treat this name as a junior synonym (of N. sepultor). Sikes et al. (2002), based on morphological data alone, stated “It is clearly closely related to N. sepultor and these two taxa are probably sister species, if not conspecific.” Details and commentary on diagnostic characters and their value are given in Sikes et al. (2002). Kozminykh later conveyed to us (in litt. 1998) that he is convinced that N. confusus is distinct from N. sepultor based on ecological work he conducted with this species in the Republic of Georgia. Kozminykh confirmed that what he considers valid N. confusus occurs in Turkey while N. sepultor does not. Our DNA samples of N. confusus were obtained near Erzurum, Turkey and their morphology agrees with the few diagnostic characters proposed for N. confusus, so there is no question that our samples belong to the taxon Kozminykh considers “true” N. confusus.

Although we have only two samples of N. confusus both are from Turkey and only a single sample of N. sepultor, from the Czech republic, the mtDNA phylogeny shows both have very shallow divergences (7–11 base pairs difference out of 2129 bp) and paraphyly of N. confusus with respect to N. sepultor. The genetic divergence between these two “species” is the smallest yet found in the genus. This, combined with a lack of adequate morphological diagnostic characters based on the examination of 288 specimens (Sikes et al., 2002), suggests that at least the N. confusus population in Turkey should be considered conspecific with N. sepultor. There remains the possibility that N. confusus is valid but has not been properly diagnosed. The holotype of N. confusus, which we have studied, is from the Xinjiang province of China, not Turkey. However, given our results we feel confident that the younger name, N. confusus Portevin 1924 is a junior synonym of the senior name, N. sepultor Charpentier 1825 (NEW SYNONYMY). More thorough sampling throughout the range of these two species, especially at the type locality in China, should help confirm or reject our conclusions here.

4.5.4. Nicrophorus mexicanus and N. nigrita

These two species are parapatric and among the closest genetically so far recorded within the genus—they range from 0.71% to 1.13% distant based on the combined COI and COII sequence data (uncorrected). The few samples of these species we have obtained so far form reciprocally monophyletic groups corresponding to the named species (Fig. 5) with moderate support (75% and 86% ML bootstrap). The average distance between species in the investigator species group is ~7%, far larger than the distances seen between these two species. When the most closely related species are removed, the minimum distance among species within the species group is ~2.0%, still larger than the distances seen for this pair.

These species differ morphologically only in the coloration of the elytra—N. mexicanus is fully maculated in the typical bifasciate condition for the genus whereas N. nigrata lacks dorsal maculations. One additional morphological difference may exist in the larvae—there is an un sclerotized suture at the base of the urogomphus which is complete in N. nigrata but incomplete medially in N. mexicanus (Palestrini et al., 1996). This character requires further study.

Their distributions are mostly allopatric, but show instead a parapatric distribution with some possible overlap around the New York mountains of southeastern California (Fig. 2D and E). There is no obvious intergradation of elytral color in this region, although few specimens from this region have been seen. We have only seen one specimen from this region (stored in LACM) with highly reduced fascia and thus questionably intermediate between these two species (and therefore, currently unidentifiable).

Further, extensive sampling of these species throughout their ranges, especially in the small area of overlap in southeastern California, would provide a more rigorous test of the monophyly of these species. Such closely related species are ideal targets to investigate mechanisms of speciation in the genus—a currently unexplored avenue of research. This is a project we are currently pursuing. Breeding studies might show these species possess intrinsic barriers to gene flow, or not, in which case, N. mexicanus Matthews 1888 might best be classified as a subspecies of N. nigrata Manneheim 1848. Until we have completed these additional studies we retain species status for this pair.
4.5.5. N. investigator and N. interruptus

Khatichkov and Popov (2006) made a number of changes to the taxonomy of *Nicrophorus* based on their study of the male and female genitalia and use of what appears to be pre-cladistic, “evolutionary taxonomy” classification methods. Only one of their changes is relevant to the analyses of our current study—their decision to subsume *N. interruptus* under *N. investigator* as a subspecies. Our phylogenetic and morphometric results strongly reject their conclusion so we therefore return *N. interruptus* to valid species status (NEW STATUS). This very strong rejection of their results consequently throws into doubt all of their findings.

In conclusion, the presence of these pairs of closely related species, whose genetic distances overlap within-species distances (Fig. 8), suggests the investigator species group is continuing to radiate. This species group is the most species-rich in the genus and clearly contains a number of “young” species. Deciding whether a species is “young” or “incipient” can be challenging, as we have experienced during this investigation. Nonetheless, we have submitted a number of taxonomic hypotheses to rigorous testing and begun to clear the way for future research on the evolutionary history of and speciation in the genus *Nicrophorus*.

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