

Population Genetics and Phylogenetics of the Endangered American Burying Beetle, *Nicrophorus americanus* (Coleoptera: Silphidae)

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ABSTRACT The burying beetle *Nicrophorus americanus* Olivier is an endangered species known to occur in disjunct populations in 6 states. Parsimony and maximum likelihood analysis of the nuclear ribosomal DNA first internal transcribed spacer (ITS1) sequences from 10 Nicrophorinae species revealed *N. americanus* to form a distinct clade with *N. orbicollis* Say. Genetic variation within and among 5 *N. americanus* populations, collected from South Dakota, Nebraska, Oklahoma, Arkansas, and Rhode Island, was studied. Ribosomal DNA ITS1 sequences from 14 beetles revealed 48 polymorphic and 20 informative nucleotide sites. *N. americanus* genetic divergence was between 0.16 and 4.76%. We found little evidence that these 5 populations have maintained unique genetic variation. No nucleotide sites were found that were diagnostic for any of the 5 populations examined, indicating that these populations may not be necessarily treated as separate, independent objects of conservation. However, further genetic investigation is warranted before translocations are attempted among the remaining populations of the American burying beetle.

KEY WORDS *Nicrophorus americanus*, ITS1, phylogeny

THE HISTORICAL RANGE of the burying beetle *Nicrophorus americanus* Olivier once included 35 states in eastern and central United States and 3 Canadian provinces (Peck and Kaulbars 1987). By 1989, its known range was limited to Nebraska, Oklahoma, Arkansas, and Rhode Island. Because of its disappearance throughout much of its range, *N. americanus* was listed as a Federal endangered species in 1989 (Federal Register 1989). Since 1989, *N. americanus* has been found to be more widespread in Nebraska than previously believed (Ratcliffe and Jameson 1992, Bedick 1997) and was rediscovered in South Dakota and Kansas.

Although most of the New World *Nicrophorus* species have been grouped into 1 of 4 clades (species groups) by Peck and Anderson (1985), *N. americanus* did not resolve as a member of one of these groups. Thus, the closest extant relative of *N. americanus* remains unknown.

Information on how genetic variation is partitioned among remaining populations of an endangered species can be used in designing recovery programs (Vogler et al. 1993). A previous study using randomly amplified polymorphic DNA-polymerase chain reaction (PCR) (RAPD-PCR) to detect genetic variation within and between Rhode Island and Oklahoma-Arkansas populations revealed little genetic variation within and between populations (Kozol et al. 1994).

Comparable levels of genetic variation were detected in a sympatric species, *N. orbicollis* Say, based on satellite DNA repeat sequence variation (King and Cummings 1997).

Another molecular marker, the nuclear ribosomal intergenic transcribed spacer (ITS1) has proved useful for the analysis of phylogenetic relationships among closely related invertebrate taxa and populations (Vogler and DeSalle 1994, Cherry et al. 1997, Powers et al. 1997, Adams et al. 1998). We investigated the utility of the ITS1 region for determining the phylogenetic relationships of *N. americanus* to other *Nicrophorus* spp. and to determine the amount of genetic differentiation among 5 disjunct *N. americanus* populations.

Materials and Methods

Nicrophorus americanus were collected from South Dakota, Oklahoma, and Arkansas in 1998 using baited pitfall traps (Ratcliffe 1996) in full compliance with a permit issued to A.L.S. and M.F. by the U.S. Fish and Wildlife Service (Table 1). Samples were pinned or preserved in 70% ethanol. The Rhode Island *N. americanus* samples were obtained postmortem from a colony run by the Roger Williams Park Zoo, Providence, RI, with a permit issued to D.S.S. by the U. S. Fish and Wildlife Service. The other Nicrophorinae taxa were obtained from baited pitfall traps (Table 1). Voucher specimens of each species have been placed in the collection of the University of Nebraska State Museum, Lincoln, NE, or at the Department of Ecology

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Table 1. Specimens used in this study

Species	Origin	Abbrev.	Year collected	n
<i>Ptomascopus morio</i>	Kyoto, Japan		1998	1
<i>Nicrophorus americanus</i>	Lincoln Co., NE	NE	1996	1
	Block Is., RI	RI	1997	2
	Tripp Co., SD	SD	1998	3
	Muskogee Co., OK	OK	1998	4
	Sebastian Co., AR	AR	1998	4
<i>N. hybridus</i>	Calgary, AB, Canada	—	1998	1
<i>N. marginatus</i>	Pawnee Co., NE	—	1995	1
<i>N. nigrita</i>	Monterey Co., CA	—	1998	1
<i>N. orbicollis</i>	Pawnee Co., NE	—	1995	1
<i>N. quadripunctatus</i>	Kyoto, Japan	—	1998	1
<i>N. sayi</i>	Windham Co., CT	—	1998	1
<i>N. tomentosus</i>	Windham Co., CT	—	1998	1
	Pawnee Co., NE	—	1998	1
<i>N. vespilloides</i>	Hokkaido, Japan	—	1998	1

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DNA was extracted from individual legs using the chloroform-phenol technique outlined in Taylor et al. (1996). Approximately 200 bp of the 18S gene, the

entire ITS1 and a 5' portion of the 5.8S gene (Fig. 1) was amplified with the primers rDNA2 (5'-TTGAT-TACGTCCCTGCCCTT-3') (Vrain et al. 1992) and rDNA 1.58S (5'-ACGAGCCGAGTGATCCACCG-3') (Cherry et al. 1997). PCR was conducted per Szalanski et al. (1997). Amplified DNA was purified and concentrated using Microcon 50 centrifugal filter devices (Millipore, Bedford, MA) and sent to the DNA sequencing facility at Iowa State University (Ames) for direct sequencing in both directions. GenBank accession numbers for the DNA sequences are AF189187 to AF189211.

DNA sequences were aligned using the PILEUP program in GCG (Genetics Computer Group, Madison, WI) and adjusted manually. Unweighted parsimony analysis on the alignments was conducted with PAUP* 4.0b2 (Swofford 1998). Exhaustive searches were performed for the interspecific analysis and branch and bound searches were performed for the intraspecific analysis. In the interspecific analysis, gapped sites were excluded because of the ambiguity of their alignments. Parsimony bootstrap analysis included 1,000 resamplings for the interspecific analysis and 100 resamplings for the intraspecific analysis using the Branch and Bound algorithm of PAUP*.

To compare the parsimony results with maximum likelihood, we first found the most appropriate model for these data using the model-fitting routine of Frati et al. (1997). This involves generating likelihood scores for a single tree (the most parsimonious tree was used) based on 16 different likelihood models. The likelihood scores for this topology were then compared using a likelihood-ratio test (Sokal and Rohlf 1981). Based on this test, we chose the general-time-reversible model (with rate heterogeneity; gamma shape parameter was estimated = GTR+G model), which was not significantly different from the most complex model (GTR+I+G). Two searches were initiated—one that used the single most-parsimonious tree generated in our parsimony search as the starting tree to a TBR branch-swapping search, and a second that generated starting trees from step-wise addition.

Genetic distances were calculated with the DNA-DIST program of PHYLIP (Felsenstein 1993) according to the Kimura 2-parameter model of sequence evolution. We also examined each population for diagnostic nucleotides (sensu Vogler et al. 1993, Vogler and DeSalle 1994). This test is based on the idea that if a state is fixed within a single population and absent from other populations, then each unique population should be considered an evolutionary significant unit and hence a separate conservation unit. In this case, if one population had a nucleotide at a particular site in each sampled individual and no sampled members of other populations had that nucleotide, it would indicate that genetic diversity differed among and was being maintained within the studied populations (Kozol et al. 1994, Vogler and DeSalle 1994).

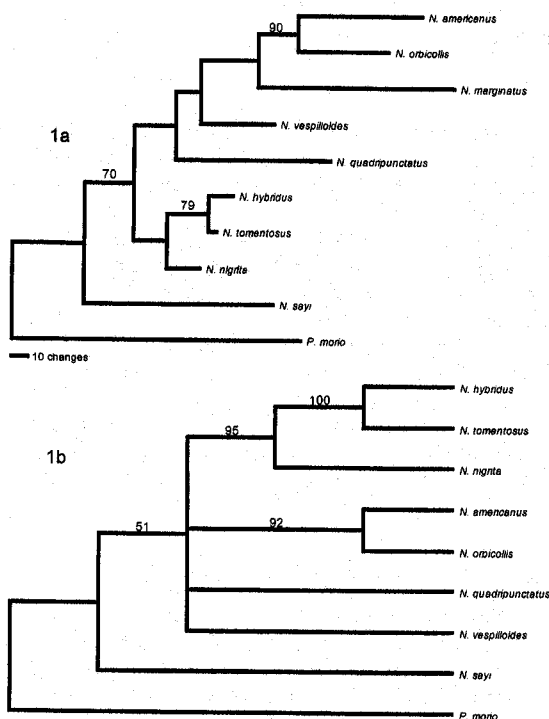


Fig. 1 (A) Single most parsimonious tree during an exhaustive search using PAUP* of 10 Nicrophorinae ITS1 DNA sequences with gapped characters excluded. Bootstrap values for 1,000 branch and bound replicates are listed above branches supported at $\geq 50\%$. (B) Majority Rule Consensus tree of 100 branch and bound bootstrap replicates run with gapped characters included and *N. marginatus* excluded.

* (and other methods).

Table 2. Nucleotide substitution rates among 14 *N. americanus* individuals

No.	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	NaOK2	—														
2	NaOK3	0.0130	—													
3	NaAR8	0.0264	0.0181	—												
4	NaSD5	0.0297	0.0197	0.0097	—											
5	NaRI1	0.0314	0.0214	0.0147	0.0081	—										
6	NaRI2	0.0298	0.0198	0.0131	0.0065	0.0016	—									
7	NaSD3	0.0365	0.0264	0.0180	0.0130	0.0081	0.0097	—								
8	NaAR2	0.0282	0.0215	0.0148	0.0146	0.0113	0.0097	0.0196	—							
9	NaAR10	0.0405	0.0285	0.0198	0.0231	0.0180	0.0164	0.0264	0.0181	—						
10	NaNE3	0.0300	0.0233	0.0216	0.0198	0.0215	0.0216	0.0266	0.0233	0.0322	—					
11	NaAR6	0.0388	0.0320	0.0250	0.0265	0.0283	0.0283	0.0334	0.0215	0.0336	0.0252	—				
12	NaSD1	0.0434	0.0315	0.0147	0.0211	0.0297	0.0280	0.0348	0.0280	0.0299	0.0299	0.0282	—			
13	NaOK6	0.0438	0.0386	0.0233	0.0299	0.0317	0.0317	0.0351	0.0284	0.0304	0.0371	0.0304	0.0351	—		
14	NaOK5	0.0476	0.0371	0.0266	0.0266	0.0265	0.0283	0.0299	0.0266	0.0319	0.0338	0.0233	0.0334	0.0320	—	
15	Norbi	0.1077	0.0948	0.0781	0.0774	0.0823	0.0799	0.0848	0.0873	0.0929	0.0834	0.0971	0.0895	0.1013	0.1030	—

Results

The rDNA2/rDNA1.58S amplicon ranged between 488 and 698 bp among the 10 *Nicrophorinae* species. With or without gapped sites included, nucleotide base frequencies did not differ among the *nicrophorine* taxa (with gaps: $\chi^2 = 24.05$, $P = 0.63$, without gaps: $\chi^2 = 4.29$, $P = 0.99$) with average base frequencies of A = 0.24, C = 0.24, G = 0.25, and T = 0.27 across the entire amplicon. The rDNA sequence was aligned for all of the *Nicrophorus* using *Ptomascopus morio* Kraatz as the outgroup taxon. The aligned DNA data matrix, including the outgroup taxon (available upon request from either of the first 2 authors, and at the web site (<http://ianrwww.unl.edu/ianr/plntpath/nematode/aszalans.htm>)) resulted in a total of 887 characters, including gaps. Of these 887 characters, 264 (30%) were variable and 120 (13.5%) were informative. With the 499 gapped characters excluded, there remained 388 characters of which 70 (18%) were variable and 40 (10%) were informative. Pairwise Tajima-Nei distances among *Nicrophorus* taxa ranged from 0.02 between *N. hybridus* and *N. tomentosus* to 0.31 between *N. marginatus* and *N. sayi*.

This dataset had only 1 most parsimonious tree (Fig. 1a), (length = 188, CI = 0.78, CI excluding uninformative sites = 0.61), as documented using the Exhaustive search algorithm of PAUP*, which examined all 2,027,025 possible trees of this dataset. There were 7 trees 1 step longer and 14 trees 2 steps longer. Bootstrap analysis of the aligned *Nicrophorus* species and *P. morio* ITS1 sequences using PAUP resulted in a consensus tree with 3 well-supported branches (Fig. 1a). *N. americanus* formed a distinct clade with *N. orbicollis* relative to the other *Nicrophorus* taxa.

Regardless of whether the starting tree was the most parsimonious tree or was obtained via step-wise addition, the GTR+C maximum likelihood (ML) search found only 1 tree. This ML tree differed from the most parsimonious tree only in the placement of *N. sayi* and *N. marginatus*; the latter was placed within the investigator group clade [(*N. hybridus* + *N. tomentosus*) + (*N. marginatus* + *N. nigrita*)]. However, the placement of *N. americanus* was identical to that of the parsimony tree. We compared likelihood scores (un-

der the GTR+G model) and tree lengths of the most parsimonious tree and the 1 likelihood tree with the Kishino-Hasegawa test and found these trees were not significantly different ($P > 0.48$). The tree favored by likelihood (length = 192, CI = 0.77) was 4 steps longer than the most parsimonious tree (length = 188, CI = 0.78).

Because parsimony analysis with gapped characters included showed lack of resolution caused by variable placements of *N. marginatus*, and the ML analysis also seemed affected by this long branch, additional analyses were conducted without *N. marginatus*. With gapped characters included, but *N. marginatus* excluded, a single shortest tree was found, bootstrap values increased significantly, and an additional branch was supported above the 50% level (Fig. 1b).

For the phylogeographic analysis, DNA sequences from 14 *N. americanus* beetles were aligned using *N. orbicollis* as the outgroup taxon. The alignment of the ITS1 DNA sequences, available at the previously mentioned WWW site, resulted in a total of 647 characters, including gaps. Of the 647 characters, 20 were variable and 19 variable characters were informative among the 14 American burying beetles. Pairwise Tajima-Nei distances among the *N. americanus* sequences averaged 0.022 (Table 2). The branch and bound search found 11 most parsimonious trees (all length = 99, CI = 0.81, CI excluding uninformative sites = 0.62) from which we generated a 50% Majority Rule Consensus tree (Fig. 2a). 100 bootstrap replicates found only 1 branch weakly supported above 50% (51%).

None of the 20 variable sites among the 14 *N. americanus* haplotypes was found to be diagnostic for any of the 5 populations examined. There were 8 potentially diagnostic states within single populations (Oklahoma, 4 sites; South Dakota, 3 sites; Arkansas, 1 site). However, none of these states was fixed within the populations and thus did not meet Vogler and DeSalle's (1994) definition of diagnostic. Of the 19 parsimony-informative sites, the Rhode Island population was polymorphic for only one 1, whereas the western populations, considered as a single unit, were polymorphic for all 19 sites.

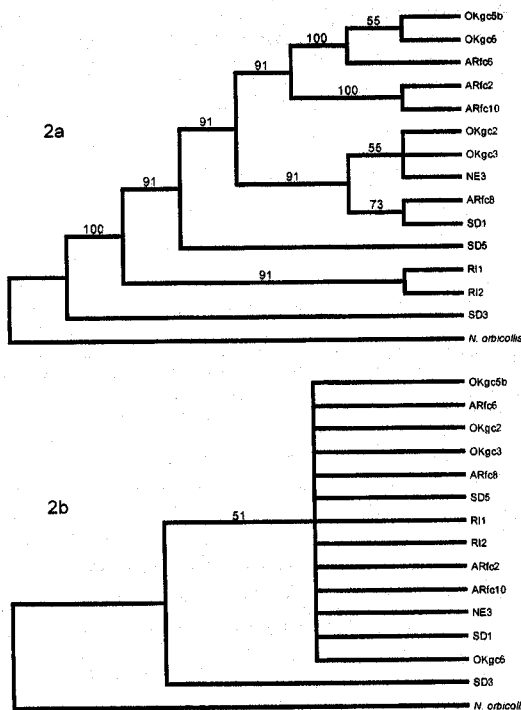


Fig. 2 (A) 50% majority rule consensus of 11 most most parsimonious trees found during a branch and bound search using PAUP* of 14 *N. americanus* and 1 *N. orbicollis* ITS1 DNA sequences. (B) 50% Majority Rule Consensus tree of 100 branch and bound bootstrap replicates.

Discussion

Based on the rDNA cladogram, the American burying beetle shares more ITS1 synapomorphies with *N. orbicollis* than other species in our dataset. However, this result does not imply that *N. americanus* and *N. orbicollis* are sister taxa in the strict sense. Morphological data (D.S.S., unpublished data) show a significant phenotypic gulf exists between *N. americanus* and all other *Nicrophorus* species, including *N. orbicollis*. This phenetic distance is cladistically represented by the presence in *N. americanus* of large numbers of autapomorphies and plesiomorphies and few to no synapomorphies. Thus, a lot of change has accumulated along the branch leading to *N. americanus*, which may be interpreted as indicating numerous close relatives of this species have gone extinct, including the true sister species. A complete analysis with all extant species (D.S.S., unpublished data) might find other species closer to *N. americanus* than *N. orbicollis*.

The other 2 well-supported branches on the tree in Fig. 1a hold together *N. tomentosus* and *N. hybridus*, a possibly true sister-species relationship. This relationship is corroborated by both mtDNA (COII) (D.S.S., unpublished data) and morphological parsimony analyses (loc. cit.) and corroborates the findings of Peck and Anderson (1985), whose morphological parsimony

analysis placed these 2 species together. These species and *N. nigrita*, which clusters with *N. tomentosus* and *N. hybridus* in a well-supported clade of the bootstrap tree in Fig. 1b, are the only members in this dataset that belong to the *investigator* group (sensu Hatch 1928, Peck and Anderson 1985).

The other taxa in this analysis are single representatives of larger species groups (e.g., *N. quadripunctatus* is just 1 of 13 species in the *nepalensis* group endemic to Eastern and Southeastern Asia). The relationships among these groups have never been carefully examined by prior authors, and preliminary results of D.S.S. (unpublished data) are currently too equivocal to compare with our current results.

The results of the maximum likelihood search help corroborate the relationship of *N. americanus* and *N. orbicollis*. However, the placement of *N. marginatus* within the *investigator* group conflicts with all prior published and unpublished analyses. This result is perplexing. *N. marginatus* and *N. sayi* are the longest branches on the maximum likelihood tree and it is known that both parsimony and maximum likelihood can fail, in certain cases, to recover the correct topology because of the presence of overly long branches. In the case of parsimony, this is the well known "Felsenstein Zone" or the phenomenon of "long branch attraction" (Felsenstein 1978, Hendy and Penny 1989), and in the case of maximum likelihood, this recently discovered inconsistency has been dubbed "long branch repulsion" or the "Farris Zone" (Siddall 1998). However, because the parsimony tree does not join these two long branches, this is probably not a case of repulsion. It seems more likely that this is a case of insufficient data or misalignment leading to an incorrect topology, an argument supported in part by the increased bootstrap results when this branch is removed from the matrix (Fig. 1b).

Knowledge that *N. orbicollis* is phylogenetically close to *N. americanus* might help to explain aspects of the ecology of these species, especially their competitive interactions. The historical range, and presumably the ecological tolerances, of *N. americanus* are most similar to 3 other Nearctic congeners—*N. orbicollis*, *N. tomentosus*, and *N. pustulatus*. However, of the latter of these, *N. tomentosus* is a late summer to fall breeder and doesn't compete as directly for carcasses with *N. americanus*, which breeds during the summer as does *N. orbicollis*, and *N. pustulatus* appears to rarely use carcasses. *N. pustulatus* is rarely captured in standard baited pitfalls (Trumbo 1990, Robertson 1992) and although it breeds on carcasses in the laboratory, recent discoveries indicate it might specialize as a parasitoid of snake eggs in the wild (P. Weatherhead in litt. and submitted). Thus, the *Nicrophorus* species that appears to overlaps the greatest with the ecological niche of *N. americanus* is also the species most closely related to *N. americanus* in our dataset.

This similarity in ecological niche (geographic range, habitat preference, diel periodicity, breeding season, and so on) and new information about these species' phylogenetic relationship suggests that *N. americanus* and *N. orbicollis* are likely each other's

greatest congeneric competitors. Numerous ecological studies have documented that *N. orbicollis* is the among most common *Nicrophorus* species in traps during the breeding season of *N. americanus* (e.g., Kozol et al. 1988, Amaral et al. 1997). However, there has been little attention paid to the intensity and effects of competition between these 2 species because this might relate to the decline of the American burying beetle. If competition with *N. orbicollis* has been a significant factor in this scenario, it may have taken various forms.

These species prefer different-sized carcasses with minor overlap in carcass size. If the resources of the size preferred by *N. americanus* became limiting, competition for the shared portion between these species would increase. Change in resource availability often has been considered in explanations of the American burying beetle's decline (Amaral et al. 1997), but the potential resulting change in competition pressures between *N. americanus* and *N. orbicollis* has received less attention. Although *N. americanus*, as a result of its larger size, is known to dominate interference competition events (Kozol et al. 1988), there are unpublished data indicating *N. orbicollis* may dominate exploitative competition events (C. Y. Matthews, in litt.) and thus contribute to the current pressures affecting *N. americanus*.

However, competition between these 2 species may not be as important now as it once was. The preference for larger carcasses by, and consequently the larger body size of, *N. americanus* may have resulted, at least in part, from niche partitioning caused by past competition with *N. orbicollis* or similar, close relatives. Vertebrates, flies, and ants are known to be important competitors for carrion in burying beetle communities (Scott et al. 1987, Sikes 1996), and larger carcasses are generally harder to conceal and co-opt than smaller carcasses and also are more valuable resources to carrion scavengers. Thus, the need for larger carcasses by *N. americanus* places this beetle species in more direct competition with vertebrate scavengers than other *Nicrophorus* species.

We observed higher levels of genetic variation (3–4%) in this species than were documented in previous studies (Kozol et al. 1994, King and Cummings 1997). Kozol et al. (1994) using RAPD-PCR, observed minimal variation within or between the Block Island, RI, and Oklahoma-Arkansas populations. Populations that are differentiated from one another, based on diagnostic genetic characters, may be recognized as distinct and significant conservation or evolutionary units (Vogler et al. 1993, Vogler and DeSalle 1994). For example, Vogler and DeSalle (1993) documented that the Martha's Vineyard population of the Northeast Beach tiger beetle, *Cicindela dorsalis* Say, was diagnosable and thus deserved separate conservation status from the mainland populations.

Although some gene flow among the western *N. americanus* populations might occur, no gene flow between the Block Island and the western populations is possible. This lack of gene flow predicts differentiation should occur (Franklin 1980, Hamrick et al.

1991), yet this study has failed to document significant genetic differentiation. It is possible there has not been sufficient time since isolation. Although *N. americanus* is an endangered beetle species with a relictual population on a New England island, like *C. dorsalis*, our findings indicate that any single population of *N. americanus*, among the 5 we examined, may not be necessarily treated as separate, independent objects of conservation. These results corroborate the findings of Kozol et al. (1994).

The Block Island population, which has been used for local introductions in New England (Kozol et al. 1994) appears to have less nucleotide variation than the other populations. The Block Island DNA sequences were polymorphic for only 1 of 19 parsimony-informative sites compared with the western populations which, when examined as a whole, were polymorphic for all 19 sites. No potentially diagnostic states were found in the Block Island population, although 8 such states were found in 3 western populations. In addition, King and Cummings (1997) found low DNA sequence variation in satellite DNA repeats from burying beetles from Block Island. However, the variation observed by these authors (loc. cit.) was low (0.18–1.22%) for all three species of *Nicrophorus* they examined, relative to most insects (1–15%), and in fact, *N. americanus* had greater variability than *N. orbicollis*.

Although these results indicate that little genetic differentiation exists among the remaining populations of the American burying beetle, we found that the Block Island population appears to have less variation than the western populations. We suggest that further genetic investigation be conducted before translocations among these populations are attempted.

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