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SPECIATIONAL HISTORY IN A DIVERSE CLADE OF HABITAT-SPECIALIZED SPIDERS (ARANEAE: NESTICIDAE: *NESTICUS*): INFERENCES FROM GEOGRAPHIC-BASED SAMPLING

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Abstract.—This paper summarizes the results of an initial effort to reconstruct the speciational history of cave spiders (*Nesticus*) from the southern Appalachian Mountains of eastern North America. The Appalachian *Nesticus* fauna includes a large series of about 30 species distributed across islandlike cave and montane habitats. Many of the species are geographically restricted; all of the species are found in allopatry. Observed patterns of morphological variation and biogeographic evidence suggest that species diversification in this lineage may have occurred recently, perhaps in response to Pleistocene climatic fluctuations. To address questions about the spatial and temporal dynamics of *Nesticus* speciation, while accounting for potential phylogenetic difficulties, I have gathered nuclear and mitochondrial DNA sequences for a sample of individuals from 81 populations representing 28 *Nesticus* species. Analyses of these data indicate that considerable genetic divergence exists within and among currently recognized morphological species. Consistent with relatively deep species divergences, most of which likely predate the Pleistocene, is a prevailing pattern of phylogenetic concordance between taxonomic species and monophyletic gene tree lineages. The few deviations from monophyly detected can be tentatively attributed to a peripatric mode of speciation. Although species limits as inferred by the molecular data are generally concordant with patterns of morphological continuity and discontinuity in genitalia, there is evidence to suggest that cryptic phylogenetic lineages exist within some morphologically continuous units. This observation, in combination with the general depth of species lineages, makes any argument about rapid evolution in *Nesticus* genitalic characteristics unnecessary.

Key words.—Biogeography, geographic variation, mitochondrial DNA, nuclear ribosomal DNA, phylogeny, speciation, spider genitalia.

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Understanding how organismal phenotypes vary across geography both within and among species has long played a key role in the study of adaptation and speciation (see Mayr 1942; Endler 1977). By allowing one to study both processes in the context of phylogeny, the recent influx of molecular phylogenetic information has the potential to further strengthen this geography-based approach. This potential is well illustrated by the many "phylogeographic" studies that consider patterns of geographic variation and population history in a single species (see Avise et al. 1987; Avise 1994). Although many fewer examples exist, the combination of molecular phylogenetics with a sampling strategy that considers intraspecific geographical variation can also benefit cross-species systematic analyses, especially when the analysis is focused on processes of species diversification (e.g., Melnick et al. 1993; Knox and Palmer 1995; Shaw 1996).

A fundamental reason for considering intraspecific variation, particularly when using molecular data to address species-level phylogenetic questions, relates to gene tree–species tree discordance. Under many biological conditions gene trees do not necessarily coincide with the actual evolutionary relationships of the species involved, due to introgression, selection at the nucleotide level, and/or retention of ancestral genetic polymorphism (e.g., Boyce et al. 1994; Patton and Smith 1994; Sperling and Hickey 1994). If available evidence suggests that such conditions (e.g., shallow divergences) likely apply in the group of interest, dependence upon traditional, "single individual per species," systematic sampling can often lead to uninformative and perhaps misinformative inferences about phylogenetic relationship (see Hoelzer et al. 1992; Page 1993). Geographical sampling, particularly in the context of multiple gene trees (Pamilo and Nei 1988; Takahata 1989), allows one to at least detect and perhaps distinguish between alternative causes for gene tree–species tree discordance.

Geography-based sampling also provides the foundation for testing hypotheses about speciation (see Harrison 1991; Templeton 1994). Researchers have used interspecific phylogeny combined with geographical sampling to make inferences about population size (e.g., Hey and Kliman 1993; Wang and Hey 1996), temporal dynamics (e.g., Brower 1994), and the geographic context of speciation (e.g., Neigel and Avise 1986; Brown et al. 1996). Understanding how molecular phylogenetic variation is geographically partitioned also provides a basis for clarifying species limits. For example, a relatively widespread species with geographic isolates may include divergent phylogenetic lineages not reflected in morphology or ecology (e.g., Larson 1984; Highton 1995). Conversely, a series of geographically adjacent, narrowly distributed morphospecies may actually represent recently fragmented variants of a single lineage. In both instances, geographical sampling of molecular variation complements morphological or distributional criteria, which individually are potentially conservative or misleading with respect to both the units and processes of species diversification.

Appalachian Nesticus

North American representatives of the spider genus *Nesticus* occur in mountainous regions of the southern Appala-

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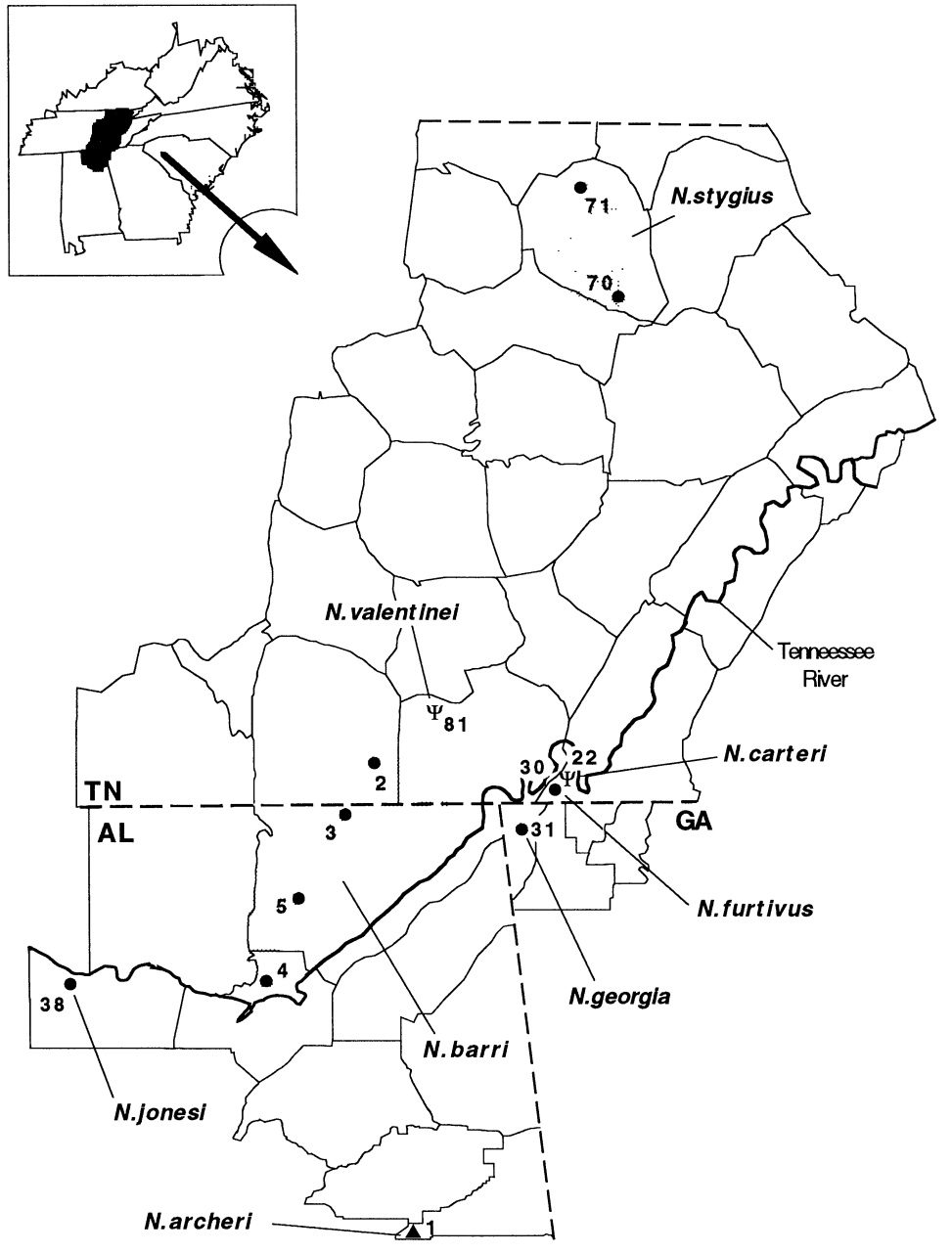


FIG. 1. Distribution of *Nesticus* in the Cumberland Plateau geologic province. Collections include 11 populations representing seven species. Collection localities are indicated by either circles (deep-cave habitats), candelabras (cave entrances), or triangles (surface habitats) with corresponding locality numbers as given in the Appendix. *Nesticus furtivus, N. jonesi*, and *N. valentinei* are known only from the type locality. The approximate distributions of *N. barri* and *N. stygius* are indicated by stippling, which coincides roughly with caverniferous limestone exposure. Note: type locality of *N. archeri* is within the Appalachian Valley and Ridge geologic province, but is figured here for convenience. This species is known only from the type locality.

chians, Pacific Northwest, and highlands of eastern Mexico (Gertsch 1984). The Appalachian fauna is the most diverse of the three *Nesticus* faunas, with at least 30 morphological species distributed primarily across the Cumberland Plateau, Appalachian Valley and Ridge, and Blue Ridge geologic provinces (Figs. 1–3). *Nesticus* build small space webs, typically in cool, moist, and dark microenvironments. At lower elevations in the Appalachian Valley and the Cumberland Plateau, *Nesticus* are found almost exclusively in caves, including both cave entrance and deep cave habitats. At higher elevations, mostly in the Blue Ridge, species are typically found in north-facing microenvironments associated with boulder fields, cove forests, and rocky gorges (Gertsch 1984; Coyle and McGarity 1992). Most species are characterized by restricted geographic distributions, often to single cave systems or mountain ranges, with seven described species known only from single populations (Gertsch 1984). Almost all species are strictly allopatric, although there are examples of different species occurring in the same mountain range or local karst system (pers. obs.). However, individuals of dif-

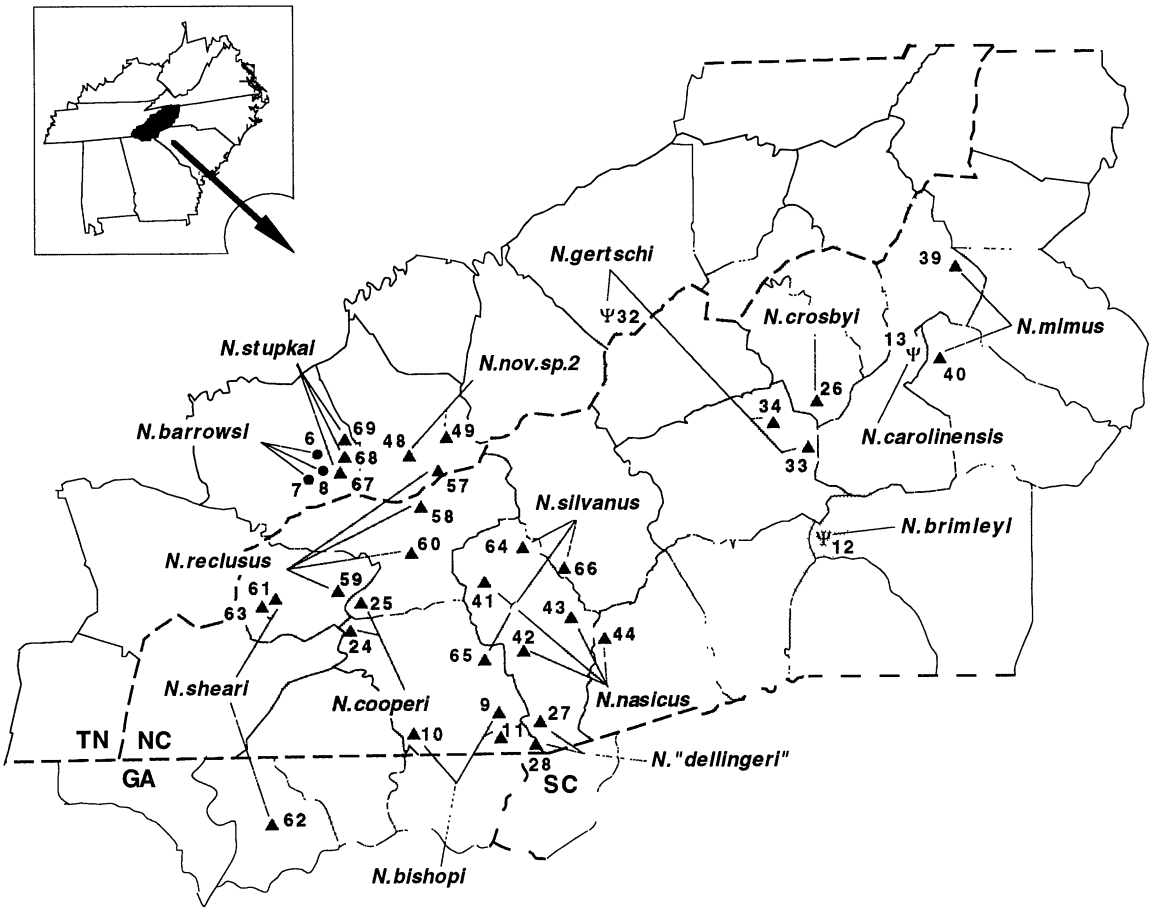


FIG. 2. Distribution of *Nesticus* in the Blue Ridge geologic province. Collections include 37 populations representing 15 species; localities are indicated by either circles (deep-cave habitats), candelabras (cave entrances), or triangles (surface habitats) with corresponding locality numbers as given in the Appendix. Stippled areas represent uplands with elevations above 600 meters. *Nesticus crosbyi* is known only from the type locality.

ferent species have never been collected in syntopy (Gertsch 1984; Coyle and McGarity 1992).

Other biogeographic information, aside from strict allopatry, suggests that much of the species diversity observed in *Nesticus* may have resulted from speciation processes that have occurred relatively recently. Gertsch (1984) hypothesized that "the series of species appearing in Appalachia was probably derived from a single basic stock that ranged widely during the early development of the group and later fragmented in response to geologic isolation and climatic pressures." Although Gertsch (1984) provided no references to support his fragmentation hypothesis, his ideas agree in principle with the "Pleistocene Effects" model (Holsinger 1988), in which habitat fragmentation events are hypothesized to have played a causal role in species diversification of cyrophylic lineages inhabiting the southern Appalachian region.

The Pleistocene Effects model is derived primarily from distributional data for terrestrial, invertebrate lineages (mostly beetles) occupying the temperate latitudes of southern Europe, Japan, and eastern North America, including the unglaciated southern Appalachian Mountains (Holsinger 1988). Although originally formulated to explain divergence processes in cave-limited taxa, the Pleistocene Effects model is also applicable to habitat-specialized montane taxa (see Kane et al. 1990). Based on a large body of biogeographic (summarized in Barr 1985; Holsinger and Culver 1985; Holsinger 1988) and paleoclimatic data (see Peck 1981), the model posits that species divergence has been facilitated by glacial-interglacial episodes of the Pleistocene. According to the model, habitat-specific, surface-dwelling taxa "track" (with respect to both elevation and latitude) favorable habitats over time, occupying more southerly and lower elevation habitats during periods of glacial maxima. Subsequently, climatic warming and the changes in the surface vegetation associated with interglacial periods induce habitat fragmentation, promoting the isolation of cave and montane taxa with the extinction or elevational retreat of surface populations.

Patterns of morphological variation in *Nesticus* are at least consistent with recent speciation. Currently recognized taxonomic species of Appalachian *Nesticus* differ most obviously in characteristics of female and particularly male genitalia, which often provide the only external characteristics available for species separation (Gertsch 1984; Coyle and McGarity 1992). This biased pattern of "species-specific" variation is consistent with the "rapid and divergent" hypothesis of Eberhard (1983, 1985), which posits that sexual

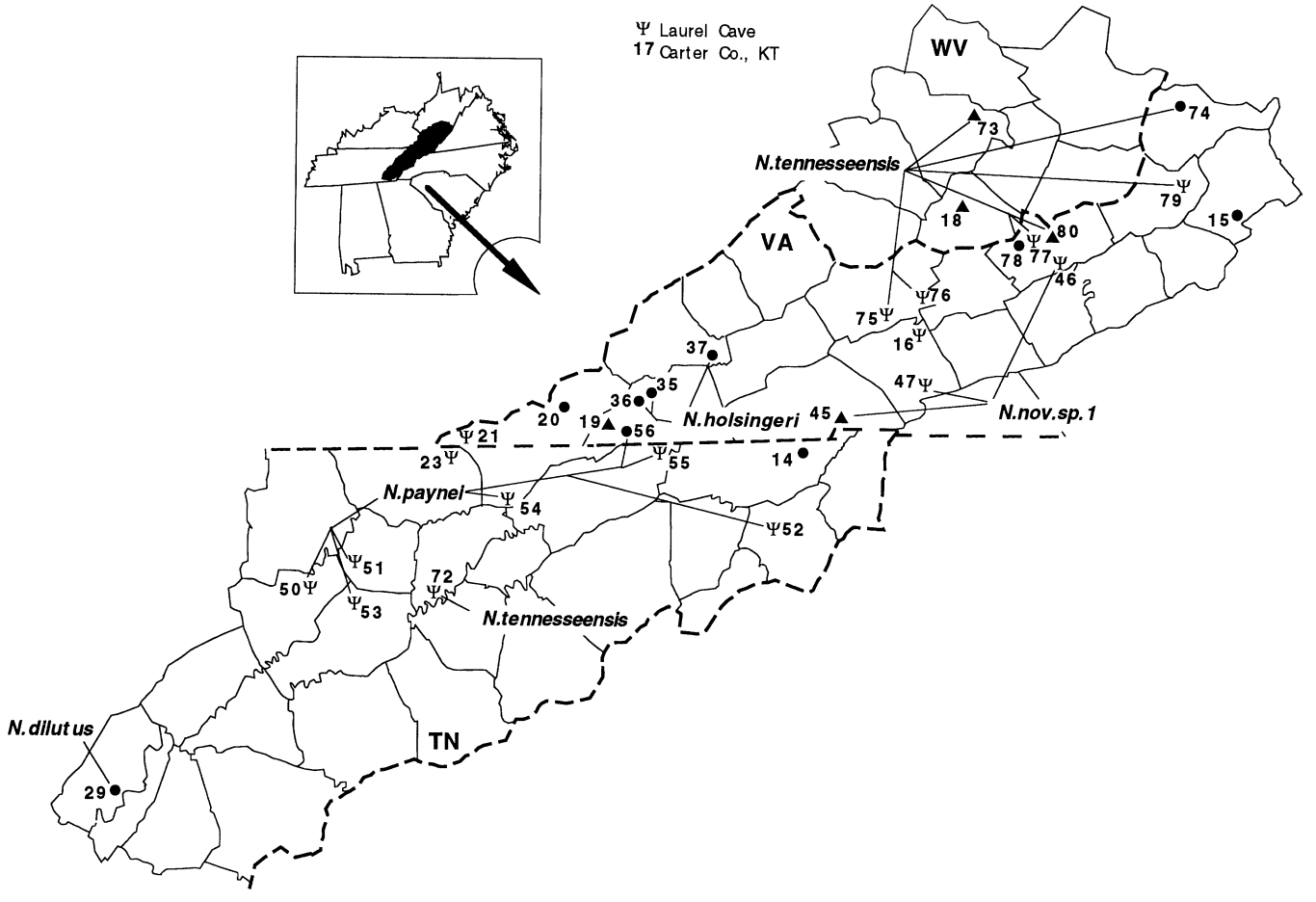


FIG. 3. Distribution of *Nesticus* in the Appalachian Valley and Ridge geologic province. Collections include 33 populations representing seven species (this total includes the type population of *N. archeri* included with Fig. 1); localities are indicated by either circles (deep-cave habitats), candelabras (cave entrances), or triangles (surface habitats) with corresponding locality numbers as given in the Appendix. Numbered populations not associated with a species label (14–21, 23) represent *N. carteri*. *Nesticus dilutus* is known only from the type locality.

selection via female choice is a strong selective force in promoting rapid divergence in secondary sexual characteristics (see also Huber 1993).

Objectives

To address questions about the speciational history of Appalachian *Nesticus* I have gathered nuclear and mitochondrial DNA sequences for a geographically comprehensive set of populations representing 28 species. Gene trees are estimated from these data using a combination of intra- and interspecific phylogenetic estimation procedures (cf. Crandall and Fitzpatrick 1996), providing a framework for comparing levels and geographic structuring of molecular variation within and between taxonomic species distributed across different geologic provinces. In combination with mitochondrial DNA molecular clock calibrations, the data are used to estimate the time frame of speciation for these species, providing a test of the Pleistocene Effects model. Finally, I examine the concordance between morphologically defined species and molecular phylogenetic lineages, particularly as this relationship relates to alternative modes of speciation and to Eberhard's (1983, 1985) rapid and divergent hypothesis.

MATERIALS AND METHODS

Sampling of Taxa

Spider populations were sampled to cover the distributional ranges of Appalachian *Nesticus* species. Except for the six species endemic to a single locality, multiple populations were sampled for all but three species known to occur in two or more localities. These three taxa (*N. brimleyi, N. carolinensis*, and *N. georgia*) are all cave-dwelling species with highly localized distributions within a single cave system (Gertsch 1984). In total, spiders from 81 populations representing 28 species (25 of 26 described; three undescribed) were collected (Figs. 1–3; Appendix). To verify that my species designations are consistent with prior diagnoses of Gertsch (1984) and Coyle and McGarity (1992), I collected representative populations from the type locality or nearby, and compared my collections with the type material for all described Appalachian species (with the exception of *N. carteri*). *Nesticus silvestrii* (a species distributed in the Pacific Northwest) and *Eidmannella pallida* (a North American nesticid) were included as outgroups, although their inclusion is not intended as a rigorous test of Appalachian *Nesticus* monophyly.

Generating Sequence Data

Juvenile and adult spiders were either preserved in the field in liquid nitrogen or 70% ethanol, or returned to the laboratory alive. Leg tissues of adult individuals were used for most DNA extractions, utilizing a modified *Drosophila* protocol (Hedin 1997).

Mitochondrial DNA Sequences.—The polymerase chain reaction (PCR) was used to amplify a 564-bp fragment spanning portions of the 16S rRNA, tRNA leucine, and NADH dehydrogenase subunit I (ND1) mitochondrial genes. PCR products were generated using the primers "N1-J-12261" 5′-TCRTAAGAAATTATTTGAGC-3′ and "LR-N-12945" 5′-CGACCTCGATGTTGAATTAA-3′, conserved oligonucleotides designed from sequence comparisons of various hexapod and spider taxa. Amplification conditions, product purification, and manual DNA sequencing methods followed Hedin (1997). Double-stranded products were sequenced directly using both external PCR primers; sequence reads averaged 300–350 bp, providing 3.5–12% overlap.

Nuclear DNA Sequences.—Double-stranded products from the nuclear ribosomal DNA ITS2 region were amplified using conserved 5.8S (5′-GGGACGATGAAGAACGCAGC-3′) and 28S (5′-TCCTCCGCTTATTGATATGC-3′) primers (White et al. 1990), with sequence modifications designed to increase specificity to invertebrates. PCR set-up and product purification was similar to that used for mtDNA amplifications. All double-stranded sequencing reactions were carried out using the *fmol* DNA Sequencing Kit (Promega), according to manufacturer's instructions. Direct sequencing using both external PCR primers provided for overlaps ranging from 50% to 75% (see below).

The direct sequencing of nuclear ribosomal DNA genes from PCR products does not allow one to adequately address the potential problem of within-individual sequence variation across the tandemly repeated transcriptional unit. This is because direct sequencing produces a consensus sequence, such that variation between repeat copies within individuals can only be detected if a particular alternative variant is relatively common. Although no such variants within individuals were detected (theoretically detectable on autorads), the possibility of within-individual variation certainly exists. However, because all intrapopulational sequence comparisons revealed no differences, and intraspecific divergences were low (see results), it is unlikely that highly divergent repeat alleles are to be found within individuals.

Phylogenetic Inference

Multiple Alignments.—Multiple alignments were used to assess site homology within *Nesticus* and to compare the structure and similarity of *Nesticus* sequences with previously published insect and arachnid DNA sequences. Alignments, performed using the programs CLUSTAL (Higgins and Sharp 1988, 1989) and TREEALIGN (Hein 1989) with default gap: change costs, were unambiguous across the low divergence comparisons considered here. Aligned sequences are available upon request from the author.

TCS Estimation Procedure.—The TCS parsimony algorithm (hereafter referred to as TCS; Templeton et al. 1992) was utilized to estimate relationships between closely related sequences, regardless of species category. This estimation procedure, although developed primarily for intraspecific data (e.g., Crandall and Templeton 1993), is also applicable at higher levels of divergence, for instance, relationships between sequences of closely related species (Crandall 1994; Templeton 1994; Crandall and Fitzpatrick 1996). Standard parsimony methods were used to corroborate results of the TCS procedure and to estimate relationships between haplotypes for which divergence levels exceeded the statistical limits of the TCS estimation procedure (see below).

Matrices of observed pairwise mutational differences between haplotypes, assuming equal weights for nucleotide changes, were calculated using the SHOWDIST command of PAUP (Swofford 1993). These matrices were used to identify groups of closely related haplotypes. Equation (8) of Templeton et al. (1992) was used to evaluate the probability P_j of a parsimonious connection (i.e., no unobserved mutations at any site) between haplotypes differing at j sites, while sharing m sites (where $j + m$ = total number of sites surveyed). For a group of closely related haplotypes, it was most efficient to consider P_j for the pair that shared the fewest common sites (m), differing at a maximum of j sites. If $P_j \geq 0.95$ (i.e., the probability of unobserved mutations is less than 0.05), maximum parsimony is justified and used to connect either two individual haplotypes that differ at j sites, or two $(j - 1)$–step haplotype networks (networks portray mutational connections between haplotypes). This step was repeated until all haplotypes were in a single network, or subdivided into two or more nonoverlapping networks. These nonoverlapping networks differed by j $(=x)$ sites, where $P_x \leq 0.95$, and therefore parsimonious connections were not statistically justified.

For the mtDNA data (but not the nDNA data; see justification below), the TCS estimation procedure was used to connect nonoverlapping networks, considering both parsimonious connections as well as connections involving additional nonparsimonious mutational steps (i.e., multiple hits). Only nonparsimonious connections involving a single inferred multiple event (reported as P_{j+1}–values) were considered in this analysis, evaluated using quation (9) of Templeton et al. (1992). All calculations were performed using the Mathematica (Wolfram 1991) package ParsimonyAnalysis provided by A. Templeton. Original papers should be referred to for full details and examples of the TCS approach (Templeton et al. 1992; Crandall and Templeton 1993; Crandall 1994).

Maximum Parsimony.—Maximum parsimony analyses utilized data matrices consisting of equally weighted nucleotide sites, with character state transformations treated as unordered and of equal cost. Most-parsimonious (MP) trees were found using heuristic searches, in which an initial tree(s) was obtained via random stepwise addition. Branch swapping on the initial tree(s) was implemented using the tree bisection-reconnection (TBR) algorithm. The above two-step procedure was replicated 10–50 times, providing alternative starting points for branch swapping. Default options for COLLAPSE, STEEPEST DESCENT, and MULPARS were in effect for these searches. Bootstrap resampling (Felsenstein 1985) was used to estimate the support for particular branch reconstructions. Parsimony and bootstrap analyses were carried out using PAUP version 3.1.1 (Swofford 1993) in combination with

MacClade version 3 (Maddison and Maddison 1992) on a Macintosh Quadra 840AV (Motorola MC68040 microprocessor) with 16 MB RAM.

Results

Mitochondrial DNA Sequence Characteristics

Mitochondrial DNA sequences were gathered for 80 of 81 Appalachian *Nesticus* populations sampled, including multiple population samples for 19 of 22 species that are known to occur in two or more localities (Appendix). All 80 sequences represent unique haplotypes. Sixty-two new (60 ingroup, two outgroup) sequences have been submitted to GenBank, with accession numbers AF004595–AF004556. New 16S and tRNA sequences supplement twenty ND1 sequences (U40499–40501, 40504, 40505, 40507–40512, 40514–40518, 40520–40523) previously reported (Hedin 1997).

Combined 16S and tRNA sequence variability is relatively conserved, with 73 of 213 sites variable across the entire array of Appalachian *Nesticus* populations. Of this total, 52 sites are polymorphic within morphologically defined species; 28 sites are potentially parsimony informative, with at least two character states each occurring in more than one taxon (Swofford 1993). Average pairwise p-distances (the observed proportion of nucleotide differences; Kumar et al. 1993) for haplotypes from populations within morphologically defined species range from 0% to 2.94% (Table 1). ND1 sequences are more variable, with 176 of 351 (50.1%) sites variable across 117 codons. Of these variable sites, 147 sites are polymorphic within one or more morphological species. The proportion of sites polymorphic at different codon positions is 37, 10, and 100 for first, second, and third codon positions, respectively; most (100/117 = 85.5%) third codon position sites are polymorphic. Average pairwise values of Kimura two-parameter distances (Kimura 1980) for comparisons within morphological species range from 1.1% to 15.6% (Table 1).

TABLE 1. Intraspecific sequence divergence values, estimated as the average pairwise proportion of nucleotide differences. All values are uncorrected for multiple hits, except mtDNA ND1 divergence values, which were estimated using the Kimura two-parameter model (Kimura 1980). ITS divergence values were calculated using pairwise deletion of gaps.

Species	mtDNA 16S and tRNA	mtDNA ND1	mtDNA ND1 transversions	ITS
Cumberland Plateau				
N. barri	0.0117	0.0632	0.0050	0.0025
N. stygius	0.0294	0.0797	0.0160	—
Blue Ridge				
N. barrowsi	0.0000	0.0193	0.0012	0.0102
N. bishopi	0.0094	0.0264	0.0035	0.0051
N. cooperi[1]	0.0118	0.0300	0.0059	—
N. "dellingeri"	0.0059	0.0131	0.0000	0.0076
N. gertschi	0.0031	0.0175	0.0024	0.0152
N. mimus[2]	0.0079	0.0201	0.0059	0.0119
N. nasicus	0.0030	0.0109	0.0012	0.0077
N. reclusus	0.0059	0.0346	0.0035	—
N. nov. sp 2	0.0118	0.0772	0.0089	—
N. sheari	0.0079	0.0247	0.0059	—
N. silvanus	0.0039	0.0636	0.0059	0.0034
N. stupkai	0.0030[3]	0.0219[3]	0.0036[3]	0.0102
Appalachian Valley				
N. carteri	0.0253	0.1559	0.0360	0.0321
N. hoslingeri	0.0079	0.0435	0.0047	—
N. nov. sp 1	0.0039	0.0402	0.0071	0.0051
N. paynei	0.0051	0.0358	0.0052	0.0076
N. tennesseensis	0.0153	0.0496	0.0047	0.0085[4]

[1] Includes *N. reclusus* 59.
[2] Includes *N. carolinensis*.
[3] Includes *N. reclusus* 58.
[4] Includes *N. dilutus*.

Phylogenetic Results: mtDNA

TCS Estimation Procedure.—Nineteen mtDNA haplotype networks were resolved using the TCS estimation procedure. Parsimonious ($P_j \geq 0.95$) connections were statistically justified for haplotypes that differed by up to 10 mutational differences. Consistent with the TCS model, there were no likely multiple hits at this level. Consideration of nearly parsimonious ($P_{j+1} \geq 0.95$) connections allowed the inclusion of haplotypes that differed by up to 25 mutational differences. Summed across all networks, 21 multiple hits were likely at this level, all of which occurred at unique third codon positions (i.e., no single site was observed to be homoplasious across two or more networks). Two-thirds of the homoplasious events involved transitional changes.

The 13 networks that included more than two populations are characterized by many extinct or unsampled haplotypes separating sampled haplotypes, suggesting significant divergence at this phylogenetic level (Fig. 4). Although most networks include haplotypes from populations of the same morphological species, four networks portray mutational connections for sequences from different species. Three of these imply unambiguous (i.e., not depending upon root placement) poly- or paraphyly: sequences of *N. reclusus* are polyphyletic and sequences of *N. mimus* are paraphyletic with respect to the *N. carolinensis* sequence.

Standard Parsimony Analyses.—A heuristic parsimony analysis of all 82 mtDNA haplotypes resulted in 640 most-parsimonious trees (length = 1635). The 19 haplotype clades corresponding to those resolved using the TCS procedure are recovered in all MP trees (a result confirmed by consideration of the strict consensus tree), and are supported by high bootstrap proportion (BP) values (Fig. 5). This correspondence suggests that results of the TCS and standard parsimony analyses are completely consistent.

There were 12 haplotypes within the species *N. barri* (2 and 3), *N. carteri* (15, 17, and 23), *N. novsp2* (48 and 49), *N. silvanus* (66), *N. stygius* (70 and 71), and *N. tennesseensis* (72 and 74) that were not included in TCS networks, as divergence levels exceeded the limits of the TCS estimation procedure. For these sequences, results of both parsimony and bootstrap analysis support species' monophyly. This support is strongest for *N. barri, N. silvanus*, and *N. stygius* haplotypes, which are recovered as monophyletic in all MP trees, and for which bootstrap proportion values are high (Fig. 5). *Nesticus tennesseensis* and *N. carteri* haplotypes are monophyletic in all or most (70%) of the MP trees, but bootstrap support for these clades is low (BP values less than 50%).

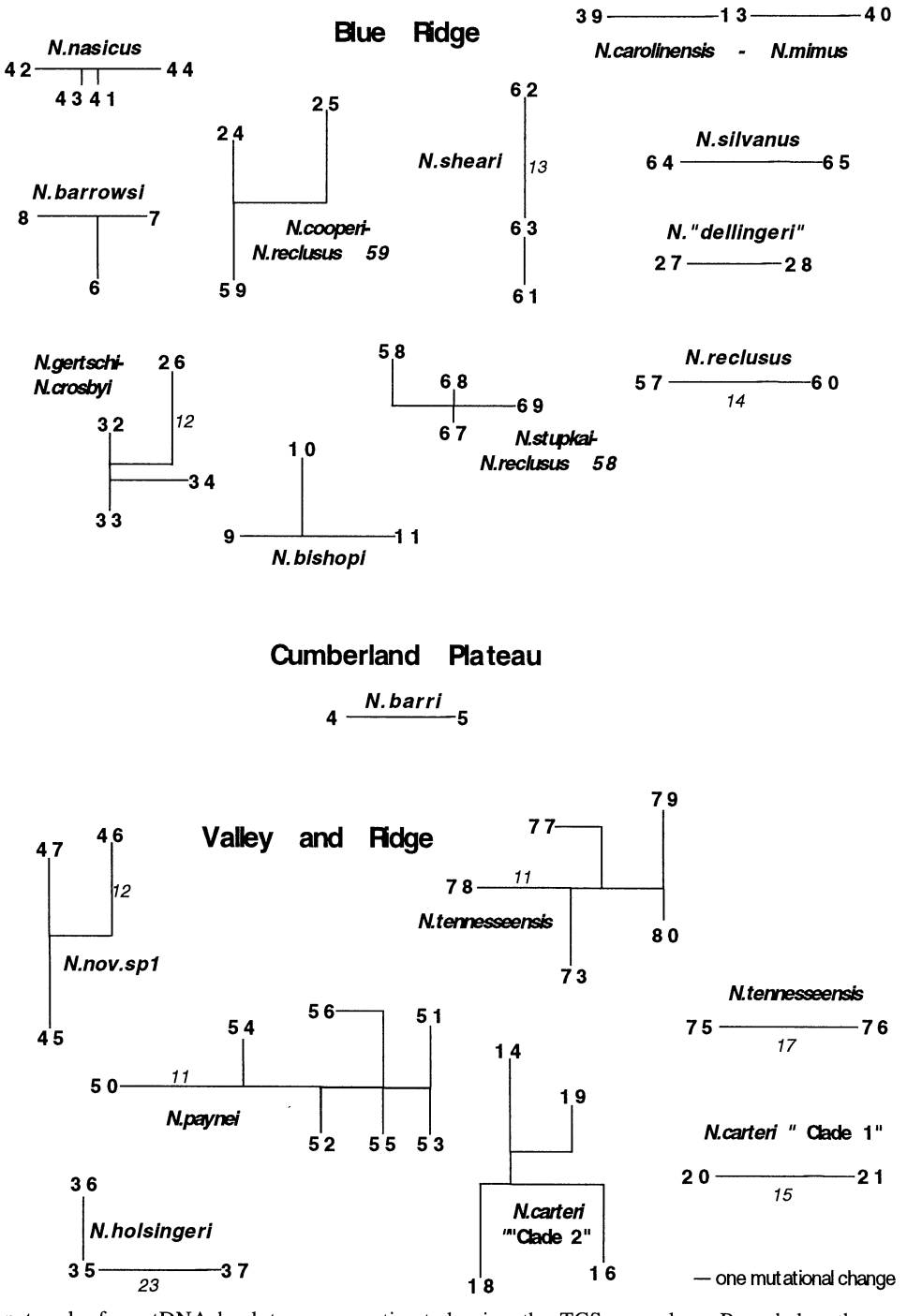
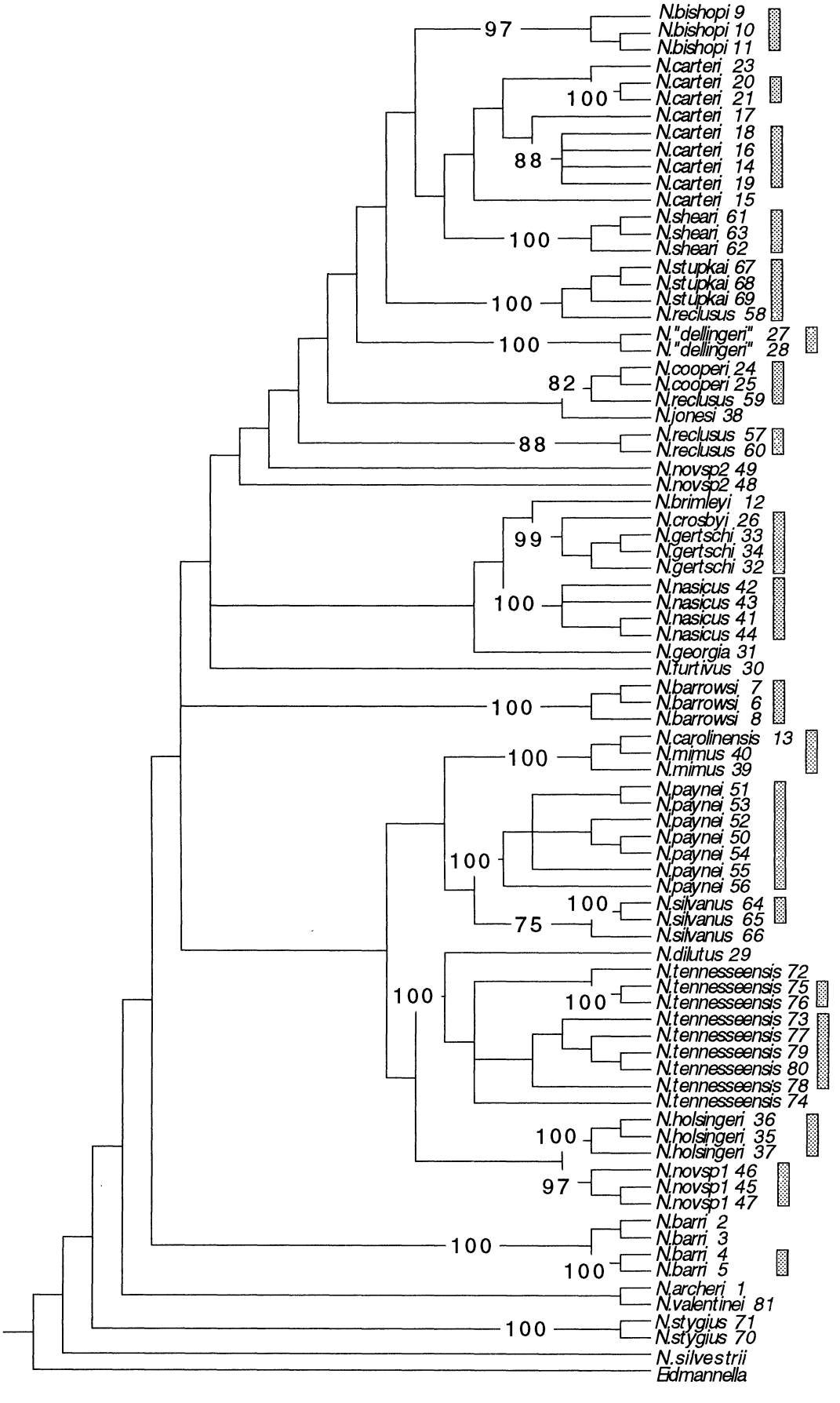


FIG. 4. Mutational networks for mtDNA haplotypes, as estimated using the TCS procedure. Branch lengths are proportional to the number of mutational steps separating haplotypes (exceptions italicized). Haplotype numbers indexed according to population origin (see Appendix).

Finally, haplotypes of *N. novsp2* are not recovered as sister taxa in any of the most-parsimonious trees. However, trees a single step longer include a monophyletic *N. novsp2*.

Nuclear DNA Sequence Characteristics

Nuclear DNA sequence data were gathered for 59 individuals from 57 ingroup populations; multiple population samples were available for only 13 of 22 species that are known to occur in two or more localities. DNA sequences sampled for multiple individuals from within populations were found to be identical, although sample sizes were limited (see Appendix). Fifty-four of 57 population sequences represent distinct haplotypes. These sequences, in addition to the *E. pallida* sequence, have been submitted to GenBank with accession numbers AF003768–AF003822.


N.bishopi 9
N.bishopi 10
N.bishopi 11
N.carteri 23
N.carteri 20
N.carteri 21
N.carteri 17
N.carteri 18
N.carteri 16
N.carteri 14
N.carteri 19
N.carteri 15
N.sheari 61
N.sheari 63
N.sheari 62
N.stupkai 67
N.stupkai 68
N.stupkai 69
N.reclusus 58
N."dellingeri" 27
N."dellingeri" 28
N.cooperi 24
N.cooperi 25
N.reclusus 59
N.jonesi 38
N.reclusus 57
N.reclusus 60
N.novsp2 49
N.novsp2 48
N.brimleyi 12
N.crosbyi 26
N.gertschi 33
N.gertschi 34
N.gertschi 32
N.nasicus 42
N.nasicus 43
N.nasicus 41
N.nasicus 44
N.georgia 31
N.furtivus 30
N.barrowsi 7
N.barrowsi 6
N.barrowsi 8
N.carolinensis 13
N.mimus 40
N.mimus 39
N.paynei 51
N.paynei 53
N.paynei 52
N.paynei 50
N.paynei 54
N.paynei 55
N.paynei 56
N.silvanus 64
N.silvanus 65
N.silvanus 66
N.dilutus 29
N.tennesseensis 72
N.tennesseensis 75
N.tennesseensis 76
N.tennesseensis 73
N.tennesseensis 77
N.tennesseensis 79
N.tennesseensis 80
N.tennesseensis 78
N.tennesseensis 74
N.holsingeri 36
N.holsingeri 35
N.holsingeri 37
N.novsp1 46
N.novsp1 45
N.novsp1 47
N.barri 2
N.barri 3
N.barri 4
N.barri 5
N.archeri 1
N.valentinei 81
N.stygius 71
N.stygius 70
N.silvestrii
Eidmannella
97
100
88
100
100
82
88
99
100
100
100
100
75
100
100
100
97
100
100

The ITS2 region comprised 319 of the 451 nucleotides sequenced for the nesticids, including the outgroup *E. pallida*. This is a consensus length that includes insertion-deletion characters required by multiple alignment. Actual ITS2 sequences ranged in length from a minimum of 282 bp for the outgroup *E. pallida*, to a maximum of 310 bp for sequences of *N. stupkai* and *N. reclusus*. The data matrix used for phylogenetic analyses consisted of 132 ingroup variable positions, 65 of which were polymorphic within morphologically defined species. Sequence divergence values at this intraspecific level were low, ranging from an average pairwise p-distance of 0.2 to 1.5% (Table 1).

Phylogenetic Results: nDNA

TCS Estimation Procedure.—Fourteen nDNA haplotype networks were resolved using the TCS estimation procedure (Fig. 6). Parsimonious connections were statistically justified ($P_j \geq 0.95$) for haplotypes that differed by up to 8 mutational steps. Because multiple hits were observed at this level, suggesting a deviation from the model underlying the TCS estimation procedure, nearly parsimonious connections were not considered. Even so, TCS networks encompassed all of the connections between haplotypes within morphological species, with the exception of haplotypes of *N. carteri*. A total of 84 parsimonious mutational events was inferred for the 14 TCS haplotype networks. These comprised 42 transitions, 33 transversions, and nine insertion-deletion events. At the network level all insertion-deletion events could be interpreted as independent, single base pair, nonadjacent events, and were therefore treated as an additional mutational class.

Standard Parsimony Analyses.—A heuristic parsimony analysis of 55 haplotypes resulted in at least 5000 MP trees (treating indels as an additional mutational class). Despite this large number of trees, haplotype clades corresponding to 13 of the 14 TCS networks were recovered in 100% of the MP trees (Fig. 7). In general, these clades are supported by high bootstrap proportion values, with the exception of the *N. stupkai* (< 50%) and *N. paynei* (52%) clades. Consistent with TCS results, sequences of *N. mimus* are paraphyletic with respect to those of *N. carolinensis* in 100% of the MP trees. Also, a majority of MP trees (60%) suggest a topology in which sequences of *N. tennesseensis* are paraphyletic with respect to those of *N. dilutus*. However, trees in which the *N. tennesseensis* haplotypes are monophyletic (with the *N. dilutus* haplotype basal) are included in the MP tree profile. As noted above, the only intraspecific haplotype relationships that were not resolved using the TCS procedure were those of *N. carteri*. These haplotypes formed a monophyletic clade in all of the MP trees (BP = 73%).

Comparisons of mtDNA and nDNA Results

If the same population samples are contrasted solely in terms of sequence divergence, nDNA haplotypes are always less divergent than mtDNA haplotypes (Table 1). In comparing gene tree topology within and between closely related species, an informative comparison requires at least three population haplotypes be sampled for each locus. There are 10 such informative comparisons possible (see nDNA networks including three or more haplotypes, Fig. 6). Six of the 10 topological comparisons, comprising haplotypes of *N. nasicus, N. gertschi, N. bishopi, N. stupkai, N. carteri* "clade 2," and the species pair *N. carolinensis* and *N. mimus*, can be made directly across TCS networks. Two of these gene tree estimates (involving *N. carteri* "clade 2" and the species pair *N. carolinensis* and *N. mimus*) do not imply the same population tree (compare Figs. 4, 6). The remaining four comparisons require consideration of both TCS and standard parsimony results (involving haplotypes of *N. barri, N. silvanus, N. carteri* "clade 1," and the species pair *N. tennesseensis* and *N. dilutus*). All of these comparisons imply the same population tree.

Discussion

Intraspecific Polymorphism, Species Divergence Times, and Biogeography

There is general evidence that morphological species of Appalachian *Nesticus* are geographically structured at the genetic level. This genetic diversity is apparent for both nuclear and mitochondrial sequences, but is more demonstrable in mtDNA sequences for which larger sample sizes are available. For example, there are no mtDNA haplotypes shared across sampling localities included in this study, regardless of the geographic proximity of populations. Although this result does not strictly apply for nDNA sequences, the majority of intraspecific comparisons imply genetic differentiation. The lower divergences observed for ITS sequences (Table 1) are consistent with expected differences in mutation rate and effective population sizes of nuclear versus mitochondrial genetic systems (Templeton 1987).

Populations of surface species distributed in the Blue Ridge province are divergent at the DNA sequence level, although relative divergence values are generally less than that observed for Cumberland Plateau or Appalachian Valley and Ridge species (at least for mtDNA; Table 1). There is no obvious single factor dictating this differentiation. For example, species that include populations on separate mountain ranges isolated by intervening valleys (e.g., *N. silvanus, N. sheari, N. bishopi*; Fig. 2) are not necessarily more polymorphic than species distributed within "contiguous" uplands (e.g., *N. reclusus, N. novsp2*). What is certain is that

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FIG. 5. Fifty-percent majority-rule consensus tree derived from 640 most-parsimonious trees resulting from a heuristic analysis of mtDNA data (tree length = 1635). Clusters that were resolved using the TCS estimation procedure (highlighted by stippled blocks) occur in all most-parsimonious trees, and are generally supported by high bootstrap values (BP > 80). An exception is the group of *N. tennesseensis* haplotypes (73, 77–80) with a BP < 50. Bootstrap proportion values based on 100 resamples of the original data matrix.

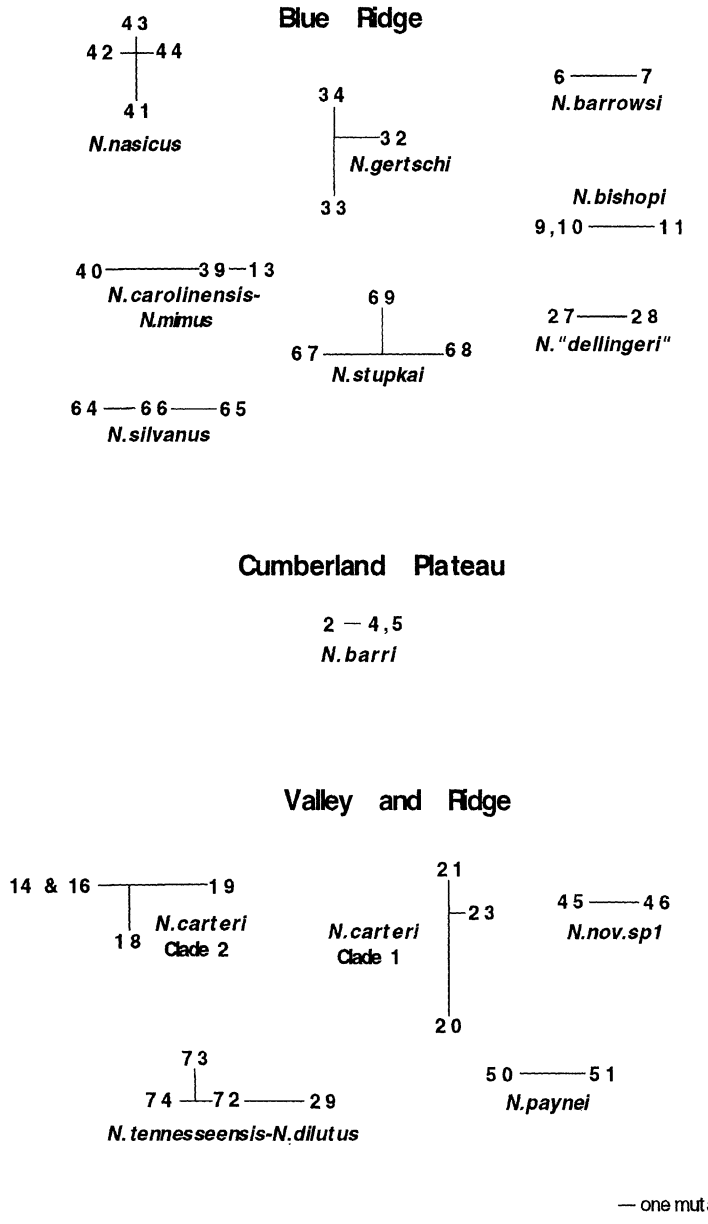

FIG. 6. Mutational networks for nDNA haplotypes, as estimated using the TCS procedure. Branch lengths are proportional to the number of mutational steps separating haplotypes (exceptions italicized). Haplotype numbers indexed according to population origin (see Appendix).

surface species of *Nesticus* are not genetically homogeneous units with respect to mtDNA or nDNA sequences (Table 1).

Most *Nesticus* populations collected from the Cumberland Plateau and Appalachian Valley and Ridge geologic provinces are probably isolated because of geographic distance, ecological and/or geological factors. Not surprisingly, intraspecific comparisons for these species reveal high levels of population differentiation, particularly for mtDNA sequences (Table 1). These divergences are high with respect to previously published arthropod mtDNA datasets, generally exceeding the interpopulation sequence divergence values of up to 5% reported for beetles and *Drosophila* (see fig. 1 of Vogler et al. 1993). Divergences also generally exceed interspecific values reported for 12S mtDNA sequences of Hawaiian *Tetragnatha* spiders (Gillespie et al. 1994), where species from separate islands within the same monophyletic group differ by 2–4%. The two sequences sampled for the obligate deep cave-dwelling species *N. stygius* (populations 70 and 71; Fig. 1) differ by at least 36 mutational differences, with a corrected distance for ND1 sequences of ca. 8% (Table 1). This value compares with uncorrected ND1 divergence values of 9% for Noctuid moths representing different *subfamilies* (Pashley and Ke 1992).

As there are no *known* divergence dates for populations or species of Appalachian *Nesticus*, it is impossible to estimate divergence times using a *Nesticus* specific molecular clock.

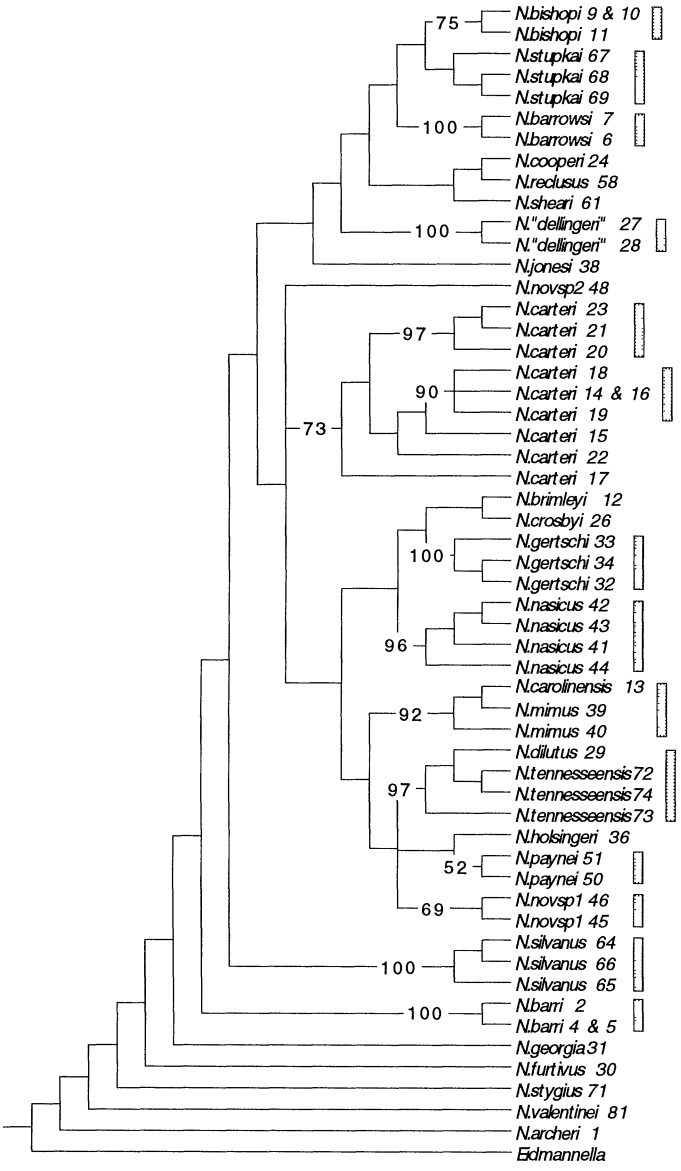

FIG. 7. Fifty-percent majority-rule consensus tree of 5000 most-parsimonious trees resulting from a heuristic analysis of nDNA data (tree length = 477; indels treated as an additional mutational class). All but one of the clades resolved using the TCS estimation procedure (highlighted by stippled blocks) was recovered in all most-parsimonious trees (*N. paynei* haplotypes were monophyletic in 64% of the MP trees). Bootstrap proportion values based on 100 resamples of the original data matrix.

However, it is possible to apply existing clock calibrations to the *Nesticus* data. I considered two different calibrations, including a ND1 calibration based on comparisons of transversional differences from dated *Drosophila* splits (DeSalle et al. 1987), and a composite mtDNA calibration estimated from closely related arthropod taxa (see table 1 of Brower 1994). I based all time estimates on intraspecific divergence values of the mtDNA ND1 data partition (Table 1), which included the largest number of variable characters. Because most of these values represent an average, rather than a maximum pairwise value for any given set of species' haplotypes, the estimates are likely to be conservative. Independent divergence values available for multiple taxa provided a crude confidence interval for these estimates.

The transversional calibration of DeSalle et al. (1987) places most intraspecific coalescence times at less than two million years ago (MYA), but this clock is expected to be variable at the low divergence levels considered here (see Templeton 1993). Brower's (1994) calibration of 2.3% pairwise divergence per million years translates into time estimates for *intraspecific* divergences ranging from a minimum of ca. 0.5 to a maximum of ca. 7 MYA (Table 1). A majority (13/19) of estimates fall in the range of 1–3.5 MYA, consistent with late Pliocene through mid Pleistocene divergences. These time depths vary across species comparisons in rough accordance with the distinction between the primarily montane species of the Blue Ridge versus the primarily cave-dwelling species of the Cumberland Plateau and Appalachian Valley and Ridge. All estimated divergence times of less that 1 MYA are restricted to montane species, perhaps suggesting that population divergence and speciation in this fauna is more recent than in the other faunas. However, the validity of this comparison across faunas is weakened by differences in the geographic scale of sampling (see Figs. 1–3) and greater potential for gene exchange across montane populations.

The late Pliocene through mid-Pleistocene time estimates suggested above are based primarily on intraspecific comparisons. This implies that the time depth of most interspecific divergences predate the Pleistocene, contrary to predictions of the Pleistocene Effects model (Holsinger 1988). Such a finding is not without precedence. As more molecular phylogenetic data are gathered for groups of species inhabiting regions thought to have been influenced by Pleistocene glaciations, it is becoming clear that many such groups have diverged much earlier than previously believed (summarized in Riddle 1996). These new findings, which apply to both temperate and tropical taxa, suggest that models of Pleistocene species divergence need to be assessed more rigorously. Even if the temporal dynamics of *Nesticus* diversification in the southern Appalachians prove atypical, the data presented here suggest the need to reevaluate the Pleistocene Effects model as it applies to other temperate, cave-dwelling lineages.

Species Monophyly

The data presented indicate that most Appalachian Nesticus species are geographically structured and polymorphic for DNA sequence variation. Nevertheless, taxonomically recognized *Nesticus* species almost always represent monophyletic lineages with respect to DNA sequences. That is, sequence coalescence events almost always occur prior to species separation events, looking backward in time. Thus the question arises as to why deviations from monophyly, particularly those resulting from the retention of ancestral genetic polymorphism, are not more prevalent. Two explanations seem most obvious.

First, variable demographic conditions prior, at, or subsequent to speciation may serve to restrict the transmission of ancestral polymorphism through population divergence and speciation (e.g., Funk et al. 1995b; Knox and Palmer 1995). Population structure analyses have shown that limited

dispersal capabilities, combined with narrow physiological tolerances, result in discontinuous population structures and relatively small population sizes in both cave- and surface-dwelling *Nesticus* (Hedin 1997). Small population size, in combination with population divergence coincident with pre-existing geographic barriers, implies that much of the lineage sorting eventually leading to monophyly may have already have been achieved prior to speciation, increasing the probability of species monophyly (Neigel and Avise 1986; Harrison 1991). However, such an explanation, although possible, is unnecessary in light of the estimated ages of *Nesticus* species lineages.

All else being equal, the time after speciation at which a pair of species is observed will be an important factor influencing genealogical status. Under neutrality, the evolutionary progression to monophyly is attributable to random extinction of sequence lineages through time, with a common time course of changes subsequent to speciation being polyphyly to paraphyly to monophyly (Pamilo and Nei 1988; Takahata 1989). Neigel and Avise (1986) have shown that, under stable demographic conditions, populations will almost always exhibit reciprocal monophyly if separation times exceed $4N_e$ generations. Assuming that most *Nesticus* species have been separated for a minimum of 500,000 generations (time estimates from above, conservatively assuming one generation per year), deviations from monophyly would require *Nesticus* mitochondrial lineages having maintained effective population sizes numbering in the hundreds of thousands. As this is unlikely, the species monophyly observed in this study most likely results from the simple fact that most Appalachian *Nesticus* species are relatively old.

Deviations from Species Monophyly

Groups of *Nesticus* populations which share qualitatively similar genital morphologies do not always represent monophyletic groups with respect to DNA sequences. Unambiguous exceptions include the species pair *N. carolinensis* and *N. mimus*, distributed in the vicinity of Linville and Grandfather Mountains in North Carolina, and a group of three species centered in the Great Smoky Mountains (including *N. reclusus, N. stupkai*, and *N. cooperi*; Fig. 2). In both instances, DNA sequences sampled from populations of one species are more closely related to sequences from populations of a second (or third) species, than they are to other "conspecific" sequences. As such, patterns of population relationship as implied by gene genealogies are inconsistent with species limits based on prior evidence (in this case, genital morphologies). As discussed below, this perceived inconsistency may or may not reflect gene tree–species tree discordance.

Because the populations involved were diagnosed in a uniform manner (i.e., using the same character systems) by the same researcher (Gertsch 1984), incorrect and/or inconsistent taxonomy cannot be used as a *general* explanation for deviations from monophyly. Introgression is also unlikely given that there are no known instances of Appalachian *Nesticus* species coexisting at a single site. Although the populations involved might have been in contact historically (e.g., see Vrba 1995), there is no available evidence to support this hypothesis. The two most probable evolutionary processes accounting for the observed poly- and paraphyly include retention of ancestral genetic polymorphism through population divergence or a speciational mode (peripatric speciation), which necessarily results in such phylogenetic patterns. Lineage sorting seems unlikely, for reasons discussed above. However, it must be noted that the species involved in deviations from monophyly are montane species distributed in the Blue Ridge province, a fauna that also includes the most recently diverged *Nesticus* species.

As more datasets that consider intraspecific sequence variation become available, it is becoming increasingly clear that peripatric speciation resulting in paraphyletic species is an important process in nature (arthropod examples include DeSalle et al. 1987; Brown et al. 1994, 1996; Funk et al. 1995a). Peripatric speciation describes the general geographic context of speciation in which a geographically restricted daughter species is derived from a more widespread parental species. Derivation of the daughter species via either microvicariance or habitat colonization can result in a phylogenetic subsampling of parental genetic variation, particularly if genetic polymorphism in the parental species is manifest as the structuring of genetic variation over geography. In phylogenetic terms, peripatric speciation results in a parental species that is paraphyletic with respect to the daughter species (Fig. 8).

Several lines of evidence support the peripatric speciation model for the sister species *N. mimus* and *N. carolinensis* (see Fig. 8). *Nesticus carolinensis*, known only from three caves in a small geographic area, is distributed peripherally to *N. mimus*, which is more widespread in adjacent montane, forested habitats. Both mitochondrial and nuclear genetic variation in *N. mimus* is geographically structured, with the variation present in *N. carolinensis* representing a subsample of that found in *N. mimus*. Furthermore, overall levels of mtDNA variation *within* populations of *N. mimus* exceed those observed in *N. carolinensis* (Hedin 1997). Finally, both mtDNA and nDNA datasets support the paraphyly of *N. mimus* with respect to *N. carolinensis* (Figs. 5, 7).

Relatively sparse nDNA geographical sampling weakens inferences regarding speciational mode for *N. reclusus, N. cooperi*, and *N. stupkai*. However, these species are also distributed in close geographic proximity, with the geographically restricted *N. cooperi* and *N. stupkai* found in montane habitats adjacent to that of the more widespread *N. reclusus* (Fig. 2). *Nesticus reclusus* is geographically structured at the mtDNA level, and haplotypes of *N. reclusus* are paraphyletic with respect to haplotypes of both *N. cooperi* and *N. stupkai* (Fig. 5). Although mtDNA haplotypes of all three species do not form a monophyletic lineage in standard parsimony analyses (resulting in a polyphyletic *N. reclusus*), trees including such a lineage are only 25 steps longer than the set of MP trees, an increase in tree length of only 1.5%.

Species, Species-Specific Genitalia, and Rapid Evolution?

Most spider species differ in characteristics of genital morphology, justifying the wide use of genitalia as diagnostic characters in species-level spider taxonomy (Coddington and Levi 1991). This biased pattern of character variation, where

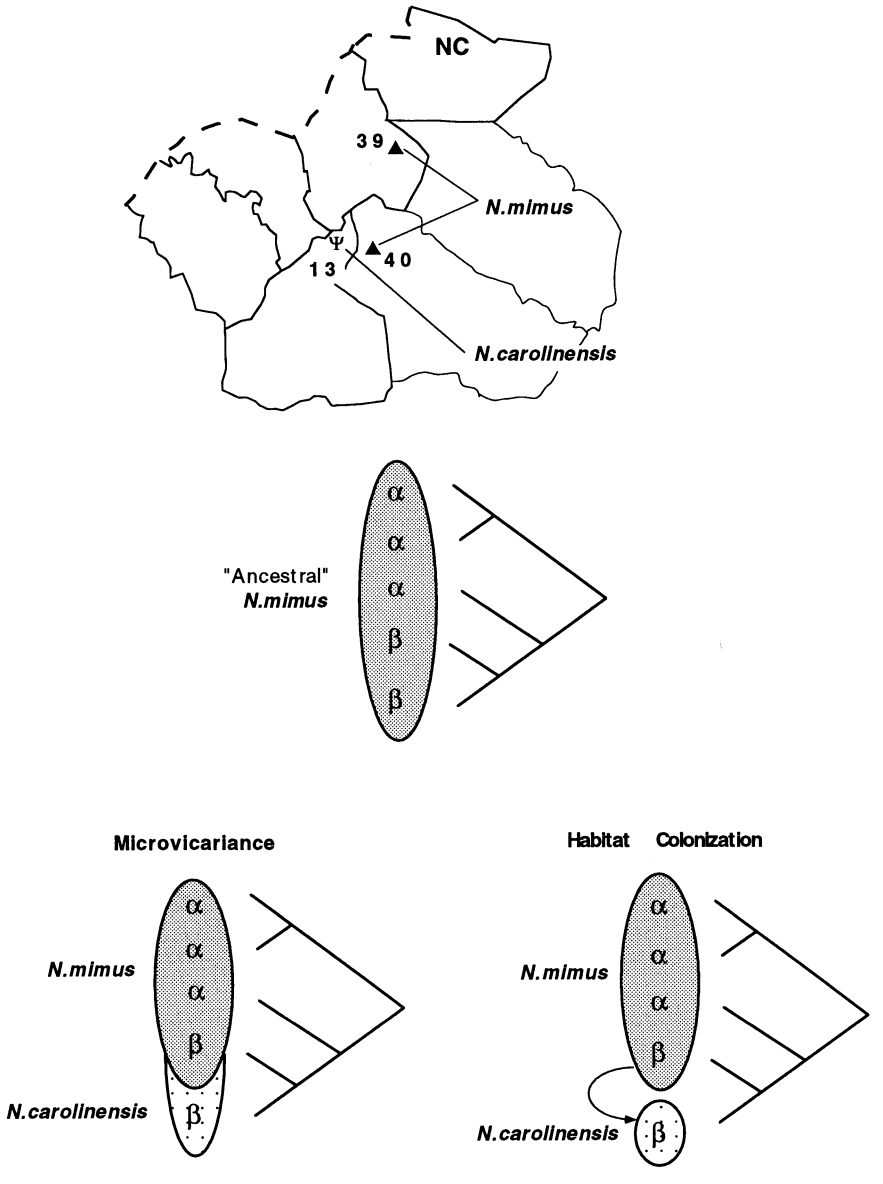

FIG. 8. Possible peripatric divergence of *N. carolinensis* via either microvicariance or a colonization event from a geographically structured ancestral *N. mimus*. Populations 13 and 40 are found in the vicinity of Linville Mountain, whereas population 39 is from the vicinity of Grandfather Mountain, North Carolina. Both mtDNA and nDNA datasets support the paraphyly of *N. mimus* with respect to *N. carolinensis*.

secondary sexual characteristics often provide the only distinguishing characters separating what are thought to be closely related species, led Eberhard (1983, 1985) to conclude that animal genitalia (or functional equivalents) evolve both rapidly and divergently, probably due to sexual selection via female choice. Superficially, divergence in Appalachian *Nesticus* agrees with the rapid and divergent hypothesis, as strictly allopatric populations (or groups thereof) of *Nesticus* spiders differ most obviously in characteristics of both male and female genital morphology (Gertsch 1984; Coyle and McGarity 1992). Functional studies of other *Nesticus* taxa (the European *N. cellanus*) provide additional indirect support for the rapid and divergent hypothesis (Huber 1993; Eberhard 1996). These *in copula* experiments reveal a complex mechanical fit between male and female genitalia, with the contacting genital structures being most divergent between closely related species.

There are, however, at least two reasons that the rapid and divergent hypothesis, as currently formulated, may not apply to Appalachian *Nesticus*. First, even though the majority of molecular lineages are concordant with species limits as recognized by patterns of continuity and discontinuity in genitalia, these species lineages are old. This finding by itself does not preclude rapid genitalic evolution, as one might hypothesize rapid, divergent genitalic evolution early in the history of a species, followed by subsequent stasis (e.g., via stabilizing sexual selection). Alternatively, one might hypothesize that genital characters are evolving in a neutral manner, with the evolution of somatic characters somehow constrained. Although these alternative hypotheses might be

distinguished (e.g., via comparative analyses of character evolution; see Lynch 1990), the important point is that the simple observation of a biased pattern of character variation alone is insufficient to make inferences about relative rates of character evolution, particularly in old taxa.

Perhaps the more convincing argument against the rapid and divergent hypothesis is the presence of cryptic lineages within morphological species of *Nesticus*. The most obvious example involves the widespread species *N. carteri*. Although the monophyly of this species is supported by both datasets, there are two geographically circumscribed, genetically divergent groups of populations that are recovered in phylogenetic analyses of both mtDNA and nDNA datasets (*N. carteri* "clades 1 and 2"; Figs. 4–7). The evidence of morphological crypsis is not restricted to this species, as both divergence and phylogenetic analyses indicate that groups of populations that share genital morphology may contain lineages that are diagnosable and divergent at the molecular level (see also Hedin 1997). These patterns suggest that divergence in genital morphologies is sometimes decoupled and conservative with respect to *Nesticus* population divergence, an observation inconsistent with predictions of the rapid and divergent hypothesis.

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APPENDIX

Locality and elevational information (in meters) for *Nesticus* and outgroup taxa. Symbols indicate specimens collected from the type locality (†), from within 5 km of the type locality (§), or from what will be the type locality of newly described species or species warranting taxonomic revision (Y). Exact microhabitat locality information is available from the author upon request. Also included are personal (MCH) lab numbers of voucher individuals sequenced for both mtDNA and nDNA genes, including redundant sequences (populations with multiple individuals with identical sequences in parentheses).

Species	Population	Elev.	mtDNA	nDNA
N. archeri	1. Cheaha Mountain SP,† Cleburne Co., AL	701	371	023
N. barri	2. Lost Cove Cave,§ Franklin Co., TN	243	065	065 (068)
	3. Salt River Cave, Franklin Co., TN	207	473	—
	4. Bishop Cave, Marshall Co., AL	305	379A	377
	5. Guess Creek Cave, Jackson Co., AL	243	380	380
N. barrowsi	6. Tuckaleechee Caverns,† Blount Co., TN	366	360	360
	7. Gregory Cave, Blount Co., TN	670	269	269
	8. Rainbow Cave, Blount Co., TN	366	281	—
N. bishopi	9. Glenn Falls,§ Macon Co., NC	945	131	131
	10. 4.3 mi. S Standing Indian, Macon Co., NC	1158	150	150
	11. Dry Falls, Macon Co., NC	1006	286	286
N. brimleyi	12. Breakdown Cave,§ Rutherford Co., NC	549	241	241
N. carolinensis	13. Linville Caverns,† McDowell Co., NC	671	222	223
N. carteri	14. Bristol Caverns, Sullivan Co., TN	518	332	332
	15. Dollhouse Cave, Rockbridge Co., VA	457	321	321
	16. Atwell's Tunnel Cave, Smyth Co., VA	701	326	326
	17. Laurel Cave,§ Carter Co., KY	213	297	296
	18. Camp Creek SP, Mercer Co., WV	670	305	305
	19. Alley Cave Entrance Sink, Scott Co., VA	457	399	399
	20. Bowling Cave, Lee Co., VA	518	340	340
	21. Skylight Cave, Lee Co., VA	549	346	345
	22. Pitchfork Cave, Hamilton Co., TN	214	—	361
	23. English Cave, Clairborne Co., TN	427	034	034
N. cooperi	24. Nantahala River,§ Macon Co., NC	792	142	142
	25. Blowing Spring, Swain Co., NC	610	683	—
N. crosbyi	26. Mt. Mitchell,† Yancey Co., NC	2012	209	209
N. "dellingeri"	27. Whiteside Mountain,Y Jackson Co., NC	1341	100	100
	28. Chattooga River, Macon Co., NC	732	109	109
N. dilutus	29. Grassy Creek Cave,† Rhea Co., TN	213	289	288
N. furtivus	30. Raccoon Valley Cvns,† Hamilton Co., TN	61	401	401
N. georgia	31. Sitton's Cave,§ Dade Co., GA	61	073	073
N. gertschi	32. Cedar Creek Cave,† Green Co., TN	427	352	351
	33. Montreat, Buncombe Co., NC	914	180	180
	34. Craggy Gardens, BRP, Buncombe Co., NC	1311	200	200
N. holsingeri	35. Pond Cave,† Scott Co., VA	500	541	—
	36. Alley Cave, Scott Co., VA	457	336	337
	37. Burton's Cave, Wise Co., VA	518	554	—
N. jonesi	38. Cave Spring Cave,† Morgan Co., AL	183	390	390
N. mimus	39. Grandfather Mountain,Y Avery Co., NC	1280	218	218
	40. Linville Gorge, Burke Co., NC	914	230	230
N. nasicus	41. Cowee Mtn. Tunnel,† Jackson Co., NC	579	188	188
	42. Wolf Creek, Jackson Co., NC	793	161	161
	43. Mull Creek, Jackson Co., NC	1097	171	171
	44. Balsam Grove, Transylvania Co., NC	1250	189	189
N. nov. sp 1	45. Neal's Sinks,Y Washington Co., VA	579	329	329
	46. Straley's Cave No. 1, Giles Co., VA	640	325	324
	47. Cow Shelter Cave, Smyth Co., VA	805	565	—
N. nov. sp 2	48. Little Pigeon River,Y Sevier Co., TN	641	262	262
	49. Chimneys, Sevier Co., TN	1006	679	—
N. paynei	50. Norris Dam Cave,§ Campbell Co., TN	305	348	348
	51. Coppock Cave, Union Co., TN	366	043	043
	52. Grindstaff Cave, Carter Co., TN	579	334	—
	53. Roaring Springs Cave, Knox Co., TN	384	483	—
	54. Cantwell Valley Cave, Hancock Co., TN	402	495	—
	55. Sensabaugh Salt Cave, Hawkins Co., TN	427	507	—
	56. Wolfe Cave, Scott Co., VA	427	520	—

APPENDIX
Continued.

Species	Population	Elev.	mtDNA	nDNA
N. reclusus	57. Newfound Gap,§ Sevier Co., TN	1506	259	—
	58. Clingman's Dome, Swain Co., NC	1963	250	250
	59. Stecoah Gap, Graham Co., NC	1036	691	—
	60. Deep Creek, Swain Co., NC	630	397	—
N. sheari	61. Joyce Kilmer Forest,† Graham Co., NC	701	093	093
	62. Sosebee Cove, Union Co., GA	1006	689	—
	63. Wright Creek, Graham Co., NC	1158	693	—
N. silvanus	64. Water Rock Knob,† Jackson Co., NC	1768	120	120
	65. Ellijay Creek, Macon Co., NC	762	395	395
	66. Steestachee Bald, Haywood Co., NC	1463	185	185
N. stupkai	67. Blowhole,† Blount Co., TN	366	285	285
	68. Little River, Blount Co., TN	305	293	293
	69. Blowing Cave, Blount Co., TN	488	356	356
N. stygius	70. Obe Lee Cave,† Overton Co., TN	396	656	—
	71. Raven Bluff Cave, Overton Co., TN	274	088	079
N. tennesseensis	72. Indian Cave,† Grainger Co., TN	305	052 (058)	052 (058)
	73. Grandview State Park, Raleigh Co., WV	732	309 (310)	309
	74. Rumbold's Cave, Alleghany Co., VA	457	313 (3140	314
	75. Fallen Rock Cave, Tazewell Co., VA	719	576	—
	76. Cassell's Farm Cave, Tazewell Co., VA	975	588	—
	77. Ballard's Cave, Giles Co., VA	549	600	—
	78. Starne's Cave, Giles Co., VA	689	611	—
	79. Walkthrough Cave, Craig Co., VA	707	633	—
	80. Little Stony Creek, Giles Co., VA	701	645	—
N. valentinei	81. Monteagle SP Cave,† Marion Co., TN	274	062	063
N. silvestrii	82. Subway Cave, Shasta Co., CA	1280	406	—
E. pallida	83. Spring Cave, Marengo, Crawford Co., IN	183	295	294