Molecular Phylogenetics at the Population/Species Interface in Cave Spiders of the Southern Appalachians (Araneae: Nesticidae: *Nesticus*)

Marshal C. Hedin

Department of Biology, Washington University

This paper focuses on the relationship between population genetic structure and speciation mechanisms in a monophyletic species group of Appalachian cave spiders (*Nesticus*). Using mtDNA sequence data gathered from 256 individuals, I analyzed patterns of genetic variation within and between populations for three pairs of closely related sister species. Each sister-pair comparison involves taxa with differing distributional and ecological attributes; if these ecological attributes are reflected in basic demographic differences, then speciation might proceed differently across these sister taxa comparisons. Both frequency-based and gene tree analyses reveal that the genetic structure of the *Nesticus* species studied is characterized by similar and essentially complete population subdivision, regardless of differences in general ecology. These findings contrast with results of prior genetic studies of cave-dwelling arthropods that have typically revealed variation in population structure corresponding to differences in general ecology. Species fragmentation through both extrinsic and intrinsic evolutionary forces has resulted in discrete, perhaps independent, populations within morphologically defined species. Large sequence divergence values observed between populations suggest that this independence may extend well into the past. These patterns of mtDNA genealogical structure and divergence imply that species as morphological lineages are currently more inclusive than basal evolutionary or phylogenetic units, a suggestion that has important implications for the study of speciation mechanisms.

Introduction

It is generally recognized that processes of speciation, similar to anagenetic processes, are influenced by population-level characteristics. For example, Templeton (1980*a*, 1980*b*, 1981) has argued that population structures in both parent and daughter species, in combination with sampling events, together play an important role in speciation. Empirical studies of both plants (e.g., Mason-Gamer, Holsinger, and Jansen 1995) and animals (e.g., Patton and Smith 1989) have confirmed these theoretical expectations, demonstrating that population genetic data are informative with respect to variation in demographic parameters prior to, during, and subsequent to species divergence. Such studies suggest that we can use information on the amount and distribution of population genetic variation in current populations to make inferences on how speciation has proceeded or is likely to proceed (Templeton 1980*a*).

Two recent developments have enhanced our ability to quantify and compare population genetic variation, particularly in a framework that reflects the historical and often hierarchical nature of speciation (Avise et al. 1987). These include (1) the ability to rapidly score DNA nucleotide polymorphism at the population level and above and (2) the concomitant growth of coalescent theory in population genetics (reviewed in Hudson 1990). DNA data not only allow us to estimate the number of alleles, their frequencies, and their geographical distributions, but they also provide information about genealogical structure. This gene tree data can be used in a coalescent framework to refine estimates of population genetic parameters such as population effective size (e.g., Felsenstein 1992) , migration rate (e.g., Slatkin 1989; Slatkin and Maddison 1989) , and population history (e.g., Griffiths and Tavaré 1994; Templeton, Routman, and Phillips 1995).

More importantly, because genealogical structure naturally extends from the population level through the population/species interface, there is a direct and complementary relationship between population genetic data and speciation mechanisms. For example, Neigel and Avise (1986) have shown through simulation that the structure of gene genealogies can be informative with respect to demographic properties (including generation time, geographic population structure and population effective size) of both parent and daughter species. We could potentially use these results to make inferences into speciation by formulating null models, deriving expected phylogenetic relationships from these models, and comparing observed relationships to those expected (Harrison 1991, fig. 2; Hey 1994). In addition, Templeton (1994) has provided empirical examples of how a phylogenetic approach that extends upward from the population level can be used to infer such processes as range expansion and colonization, vicariant fragmentation, and historical introgression. All of these processes play important roles in speciation theory.

Cave Spiders

This paper summarizes a comparative population genetic approach to the study of speciation mechanisms in a species group of Appalachian cave spiders (the *Nesticus tennesseensis* "complex," Gertsch 1984; Hedin 1995). Species of this complex are distributed in allopatry over a limited geographic area in the Cumberland Plateau, Appalachian Valley and Ridge, and Blue Ridge geologic provinces of eastern North America (fig. 1). The monophyly of the *N. tennesseensis* complex is supported in molecular systematic analyses of the entire

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Address for correspondence and reprints: Marshal C. Hedin, Department of Ecology and Evolutionary Biology, University of Arizona, Biological Sciences West, Room 310, Tucson, Arizona 85721. E-mail: mhedin@ccit.arizona.edu.

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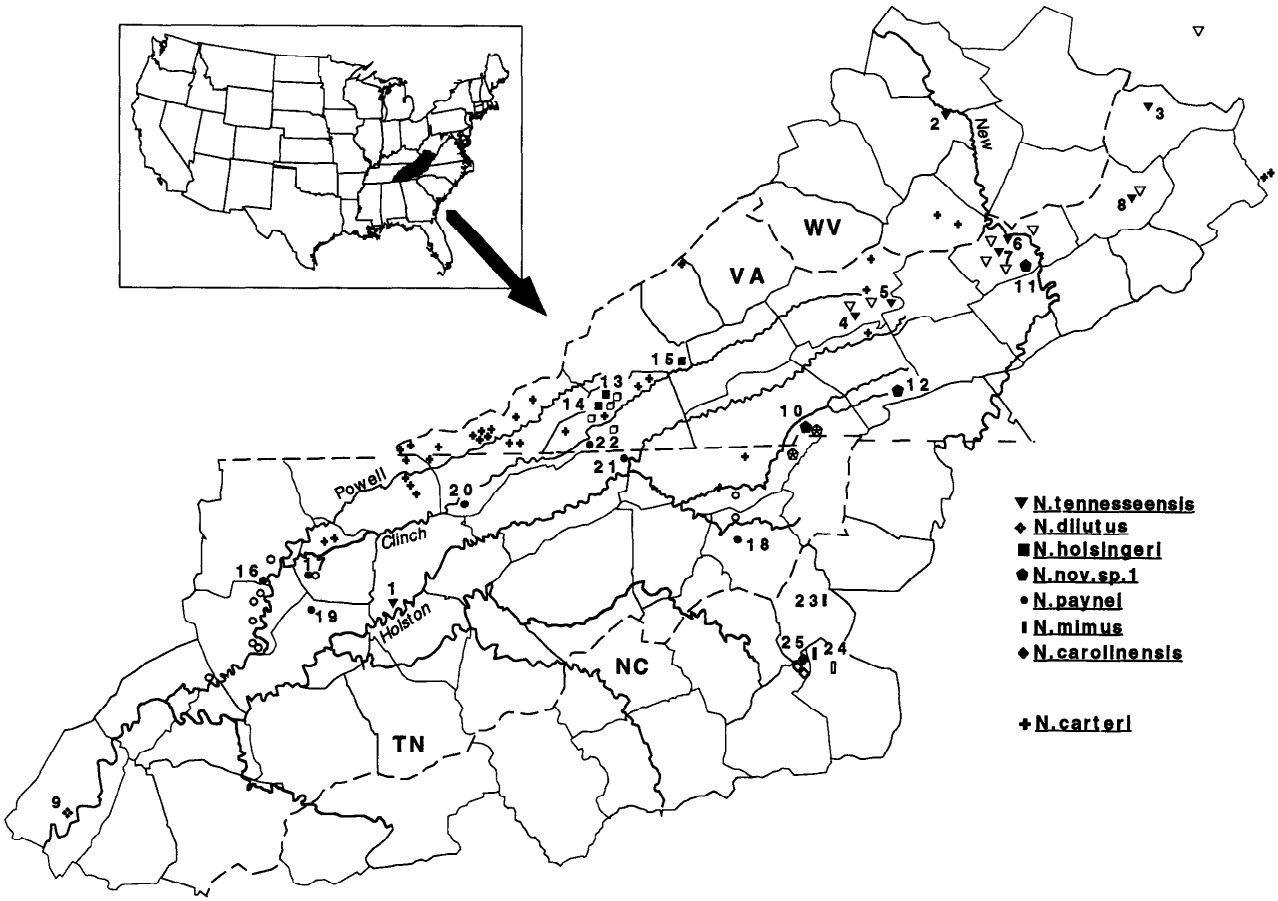

FIG. 1.—Distribution of taxa in the *Nesticus tennesseensis* species complex. Populations for which genetic data are available are indicated by filled symbols; locality numbers correspond to those given in table 1. Hollow symbols indicate known populations not included in this analysis (based on personal collections and locality information included in Gertsch [1984]). Population localities of *Nesticus carteri* are also shown. Locality information for this species is potentially important in distinguishing whether distributional gaps (for spiders in the group of interest) are due to inappropriate geology or exclusion from potentially suitable habitats.

Appalachian *Nesticus* fauna, which comprises approximately 30 species (Hedin 1995). Systematic analyses further support the recognition of three pairs of sister taxa, including *N. tennesseensis–N. dilutus, N. holsingeri–N. novspl,* and *N. paynei–N. carolinensis/N. mimus.* These taxa differ in extent of distributional area; for example, the relatively widespread *N. tennesseensis* as compared to *N. dilutus,* which is endemic to a single locality (fig. 1). In addition, the sister taxa that constitute this complex show only minor differences in the genital morphologies used to diagnose species (Gertsch 1984); the differences observed are therefore possibly a result of recent speciation.

These *Nesticus* species are also representative of the different ecological categories that have traditionally been recognized in cave-dwelling lineages (see Barr 1968; Howarth 1983). *Nesticus mimus* is a "surface-dwelling" (epigean) species, known only from high-elevation, montane habitats of North Carolina. Populations of this species have been collected in microenvironments such as north-facing boulder fields and rocky gorges (Hedin 1995). Populations of *N. carolinensis* and *N. paynei* are both apparently restricted to cave habitats (having never been recorded from epigean habitats) but are found relatively close to cave entrances, whereas *N. tennesseensis* and *N. novspl* populations are found in both cave and epigean habitats. All four of these taxa are troglophilic. Finally, *N. dilutus* and *N. holsingeri* are both morphologically specialized taxa restricted to deep-cave habitats (Gertsch 1984), and are categorized as obligate cave-dwellers (i.e., troglobites). Although best viewed as crude measures of *potential* ecological differences, these categorizations do provide a comparative framework for testing hypotheses relating to population genetic parameters and speciation processes (see also Caccone and Sbordini 1987).

I have two general objectives in this paper. I first characterize the population genetic structure of species in the *N. tennesseensis* complex, and ask whether there are differences in population structure across species in accordance with variation in habitat use. As a measure of population genetic structure, I consider the amount, distribution, and genealogical structure of mitochondrial DNA sequence variation within and between populations. All else being equal (within a lineage, over similar geology and geographical distances), I predict that populations of epigean and troglophilic species should be less structured than populations of troglobitic species,

Table 1
Collecting Localities and Elevational Information (in m) for *Nesticus* Populations

Species	Population	Drainage Basin	Elevation	Latitude/Longitude	Pop	*N*
N. tennesseensis . . .	1. Indian Cave, Grainger Co., Tenn.	Holston	305	36°09′39″N 83°36′00″W	IC	12
	2. Grandview SP, Raleigh Co., W.Va.	New	732	37°49′45″N 81°03′45″W	GV	9
	3. Rumbold's Cave, Alleghany Co., Va.	James	457	37°48′43″N 80°04′15″W	RC	12
	4. Fallen Rock Cave, Tazewell Co., Va.	Clinch	719	37°00′29″N 81°41′00″W	FR	11
	5. Cassell's Farm Cave, Tazewell Co., Va.	New	975	37°05′30″N 81°23′00″W	CF	10
	6. Ballard's Cave, Giles Co., Va.	New	549	37°19′08″N 80°43′44″W	BC	11
	7. Starne's Cave, Giles Co., Va.	New	689	37°16′56″N 80°46′58″W	SC	10
	8. Walkthrough Cave, Craig Co., Va.	New	707	37°28′40″N 80°09′18″W	WT	10
N. dilutus.	9. Grassy Creek Cave, Rhea Co., Tenn.	—	213	35°31′40″N 84°54′44″W	GC	11
N. novsp 1.	10. Neal's Sinks, Washington Co., Va.	Holston	579	36°37′43″N 81°53′16″W	NS	10
	11. Straley's Cave No. 1, Giles Co., Va.	New	640	37°15′36″N 80°38′55″W	ST	11
	12. Cow Shelter Cave, Smyth Co., Va.	Holston	805	36°46′12″N 81°24′00″W	CS	10
N. holsingeri.	13. Pond Cave, Scott Co., Va.	Clinch	500	36°45′02″N 82°42′15″W	PC	11
	14. Alley Cave, Scott Co., Va.	Clinch	457	36°42′50″N 82°43′39″W	AC	11
	15. Burton's Cave, Wise Co., Va.	Clinch	518	36°53′50″N 82°20′14″W	BU	6
N. paynei.	16. Norris Dam Cave, Campbell Co., Tenn.	Clinch	305	36°13′23″N 84°05′36″W	ND	11
	17. Coppock Cave, Union Co., Tenn.	Clinch	366	36°13′31″N 83°56′46″W	CC	9
	18. Grindstaff Cave, Carter Co., Tenn.	Holston	579	36°17′09″N 82°09′18″W	GS	6
	19. Roaring Springs Cave, Knox Co., Tenn.	Clinch	384	36°07′41″N 83°56′42″W	RS	11
	20. Cantwell Valley Cave, Hancock Co., Tenn.	Clinch	402	36°26′50″N 83°17′00″W	CV	11
	21. Sensabaugh SP Cave, Hawkins Co., Tenn.	Holston	427	36°33′57″N 82°38′05″W	SS	11
	22. Wolfe Cave, Scott Co., Va.	Clinch	427	36°38′20″N 82°45′03″W	WC	11
N. mimus.	23. Grandfather Mtn., Avery Co., N.C.	—	1280	36°05′00″N 81°51′30″W	GM	11
	24. Linville Gorge, Burke Co., N.C.	—	914	35°56′25″N 81°55′50″W	LG	9
N. carolinensis	25. Linville Caverns, McDowell Co., N.C.	—	671	35°55′10″N 81°56′15″W	LC	11
					Total	256

NOTE.—*N* is the number of individuals sampled per population; Pop designates the population acronym. Drainage basins correspond to those of Holsinger and Culver (1985).

reflecting potential differences in dispersal rate, population density, and/or ecological amplitude. This basic prediction has been confirmed, through genetic studies, in many terrestrial cave-dwelling arthropods (e.g., Caccone 1985; Crouau-Roy 1989) , including a troglophilic European *Nesticus* species. Allozyme-based estimates of gene flow are consistent with moderate to high levels of effective migration between cave-dwelling populations of *N. eremita* (Cesaroni et al. 1981; Caccone 1985) , possibly by way of airborne dispersal via silken "parachutes" (i.e., ballooning).

My second objective is to relate patterns of population genetic structure and divergence to questions about species and speciation mechanisms. As a working hypothesis, I treat species as groups of populations which share both history and genital morphologies, consistent with prior taxonomy (Gertsch 1984) and systematic studies (Hedin 1995). I explore possible reasons for tension between this morphological concept and patterns of mtDNA genealogical structure and divergence, which imply that species as morphological lineages are currently more inclusive than basal evolutionary or phylogenetic units. I conclude by considering the roles of habitat vicariance and founder event speciation in population and species divergence of Appalachian *Nesticus,* discussed in the context of available biogeographic and geologic evidence.

Methods and Materials

Population Sampling

Sampling was carried out such that populations were collected over most of each species' known geographic range, taking obvious riverine and/or stratigraphic barriers into consideration (fig. 1). Even so, there remain some marked gaps in the geographic sampling of these species. Given our current understanding of the group, it is difficult to know if such gaps represent artifacts of insufficient distributional knowledge (e.g., undiscovered populations) or if a species is lacking in certain geographic areas due to inappropriate habitat, competition, extinction, etc. However, it is important to note that the taxonomic composition and distributional patterns of invertebrate cave-dwellers in northeastern Tennessee and southwestern Virginia are among the best known in North America (see Holsinger and Culver 1985).

Spiders from a total of 25 localities were collected for voucher specimens and DNA samples (table 1). Each locality represented a discrete sampling unit, within which there were no obvious population discontinuities; spiders were collected at random from each locality. An exception was the Indian Cave population of *N. tennesseensis,* thought to be composed of two subpopulations within a single cave (Ives 1930; Gertsch 1984). In this case, exact within-cave localities were maintained to reflect a potential separation between deep-cave and cave entrance subpopulations.

Generating Sequence Data

Previously frozen, ethanol-preserved, and/or fresh specimens were used for DNA extractions. Tissues used included legs, thoraces, abdomens, or a combination of the above, depending on the size of the specimen. Tissues were taken from sexually mature individuals for a

majority of specimens. Genomic DNA was prepared using a modification of the single-fly *Drosophila* preparation (Ashburner 1989). DNA was extracted once with phenol : chloroform : isoamyl alcohol (24:24:1) and once with chloroform : isoamyl alcohol (24:1), ethanol precipitated and suspended in 50 μl distilled dH_2O.

Double-stranded products from the mitochondrial NADH dehydrogenase subunit I (ND1) gene were amplified via PCR, using oligonucleotides designed by identifying conserved regions in sequence comparisons of various hexapod and spider taxa (see appendix). The polymerase chain reaction was carried out in 50-μl volumes, using 30 μl of a 1:100 dilution of genomic DNA as template. Double-stranded reactions included 10 pmol of each primer, 100 nM dNTPs, and 2.5 mM MgCl2 *Taq* salts, using 2.0 units of *Taq* polymerase (Cetus). Reactions were run for 30 cycles, each cycle consisting of denaturation for 30 s at 95°C, annealing for 1 min at 47–50°C, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min.

PCR products were visualized on agarose gels, ethanol precipitated, and electrophoresed through 3.5% polyacrylamide gels. Gel fragments were excised and passively eluted for 24–48 h (Sambrook, Fritsch, and Maniatis 1989), ethanol precipitated, then resuspended in dH_2O to a final concentration of ~ 200 ng/μl for use in sequencing reactions. Double-stranded mtDNA templates were directly sequenced using the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (NEB), according to manufacturer's instructions. Reaction products were electrophoresed through 6% LongRanger gels (AT Biochem). One strand only was sequenced for each individual spider (sense strand); sequence types (haplotypes) found to be restricted to single individuals were sequenced twice to confirm unique variable sites.

Frequency-Based Analyses

DNA Polymorphism

The two measures of within-population DNA polymorphism considered included the number of segregating nucleotide sites per sequence (S) and the average number of nucleotide differences per sequence between all pairs of sequences ($\hat{k}$) (Nei 1987). Both measures were used to estimate the neutral parameter $\theta = 2N_{ef}\mu$, following Watterson (1975) for $\theta(S)$ and Tajima (1983) for $\theta(\hat{k})$. Here, N_{ef} is the inbreeding population effective size of females and μ is the mutation rate per DNA sequence, per generation. This estimate reflects the assumption that mitochondria in *Nesticus* show uniparental (maternal) inheritance and complete vegetative segregation.

To test whether patterns of DNA sequence polymorphism were consistent with the neutral mutation model, the D statistic of Tajima (Tajima 1989*b*) was estimated from $\theta(S)$ and $\theta(\hat{k})$ for each population. Assuming an infinite-sites equilibrium model of neutral DNA evolution, the difference between θ estimates is expected to be zero (Tajima 1989*b*). The important difference between θ estimates is the effect of selection, which can result in either significantly negative (e.g., through directional selection) or positive (e.g., through diversifying selection) D values. Nonequilibrium demographic conditions (e.g., fluctuations in population size) can also result in D values significantly different from zero, leading to a rejection of the null hypothesis (see Tajima 1989*a*; Simonsen, Churchill, and Aquadro 1995).

Population Subdivision and Divergence

Population subdivision was described using F statistics (Wright 1951), particularly F_{ST}, measured as the fraction of the total sequence diversity attributable to differences among populations. The pairwise variance approach of Hudson, Slatkin, and Maddison (1992) was used to estimate F_{ST} as 1 minus the ratio of within (H_W) versus between (H_B) population heterozygosity. In comparing two populations (X and Y), H_W is the average number of site differences between sequences sampled for all pairwise comparisons within populations, and H_B is the average number of site differences between sequences sampled from two different populations (Nei 1987).

Genealogical Analyses

Genealogical relationships of haplotypes were estimated using maximum parsimony. For each species pair, most-parsimonious (MP) trees were found using the branch-and-bound algorithm, as implemented in PAUP 3.1.1 (Swofford 1993). In these analyses, nucleotide sites were equally weighted, with character state transformations treated as unordered and of equal cost. Bootstrap resampling (Felsenstein 1985) was used to estimate the accuracy of particular branch reconstructions (see Hillis and Bull 1993).

Levels of Gene Flow

Genealogical estimates of the effective number of migrating individuals per generation (N_m) between two or more populations are generally superior to those derived from F_{ST} (Slatkin and Maddison 1989). This is not necessarily the case when gene flow is restricted to the extent that sequences from separate populations form exclusive clades, making it difficult to distinguish low rates of gene flow from nonexistent gene flow (Hudson, Slatkin, and Maddison 1992). However, Slatkin (1989) has shown that if the number of mitochondrial sequences sampled from one or more populations is reasonably large ($\geq$10), then complete concordance between the phylogenetic and geographic structure of genetic variation suggests that the average level of gene flow is likely to be relatively small ($N_m < 1$). I used Slatkin's (1989) coalescent approach to estimate upper values of N_m for population comparisons of all three species pairs.

Relative Rates

Under the nearly neutral model of molecular evolution, allelic neutrality is a function of variance in population effective size (Ohta 1976; 1992). Nearly neutral mutants are subject to drift in small populations (effectively neutral), but as population size increases, more mutations are selected against, and a smaller fraction of these reach fixation. This relationship led Ohta (1976) to the prediction that populations subjected to founder events and/or bottlenecks should display increased rates

Table 2
DNA Polymorphism Statistics

Species	Pop	#Hap	Frequency	S	$\theta(S)$	$\theta(\hat{k})$	D	SD $\theta(\hat{k})$
N. tennesseensis	IC	3	0.58, 0.25, 0.17	4	1.324	1.545	0.586	0.0040 ± 0.0029
	GV	4	0.33, 0.11, 0.33, 0.22	4	1.479	1.694	1.683	0.0045 ± 0.0032
	RC	2	0.83, 0.17	1	0.331	0.303	−0.195	0.0008 ± 0.0010
	FR	3	0.73, 0.18, 0.09	2	0.683	0.509	−0.778	0.0013 ± 0.0014
	CF	4	0.40, 0.20, 0.30, 0.10	3	1.060	1.178	1.353	0.0031 ± 0.0024
	BC	2	0.73, 0.27	2	0.683	0.873	0.850	0.0023 ± 0.0019
	SC	2	0.90, 0.10	1	0.353	0.200	−1.770*	0.0005 ± 0.0008
	WT	3	0.80, 0.10, 0.10	2	0.707	0.327	−1.734*	0.0010 ± 0.0012
N. dilutus	GC	1	1.0	0	0.0	0.0	—	0.0
N. novspl	NS	1	1.0	0	0.0	0.0	—	0.0
	ST	1	1.0	0	0.0	0.0	—	0.0
	CS	2	0.80, 0.20	1	0.353	0.360	0.015	0.0008 ± 0.0010
N. holsingeri.....	PC	3	0.82, 0.09, 0.09	2	0.683	0.360	−1.430	0.0009 ± 0.0011
	AC	2	0.91, 0.09	1	0.341	0.180	−1.128	0.0005 ± 0.0007
	BU	2	0.83, 0.17	3	1.314	1.0	−0.493	0.0026 ± 0.0023
N. paynei	ND	3	0.27, 0.55, 0.18	3	1.024	1.2	0.587	0.0032 ± 0.0025
	CC	2	0.67, 0.33	1	0.370	0.500	0.989	0.0014 ± 0.0014
	GS	2	0.83, 0.17	2	0.876	0.667	−0.410	0.0018 ± 0.0018
	RS	1	1.0	0	0.0	0.0	—	0.0
	CV	1	1.0	0	0.0	0.0	—	0.0
	SS	1	1.0	0	0.0	0.0	—	0.0
	WC	1	1.0	0	0.0	0.0	—	0.0
N. mimus.....	GM	4	0.37, 0.18, 0.36, 0.09	3	1.024	1.054	0.101	0.0028 ± 0.0023
	LG	1	1.0	0	0.0	0.0	—	0.0
N. carolinensis.....	LC	2	0.82, 0.18	2	0.683	0.654	−0.127	0.0018 ± 0.0016

NOTE.—For each population, statistics include the population acronym (Pop), the number of haplotypes (#Hap), haplotype frequencies, in numerical order (e.g., IC1, IC2, IC3), the number of segregating sites (S), $\theta(S)$ values, $\theta(\hat{k})$ values, Tajima's D values, and SD $\theta(\hat{k}) = \theta(\hat{k})$ per nucleotide site, with standard deviations calculated as the square root of the stochastic variance of the estimate (Nei 1987).

* D significantly different from 0 ($P < 0.05$); confidence limits from table 2 of Tajima (1989*b*).

of molecular evolution. DeSalle and Templeton (1988) confirmed this prediction through comparative analyses of mtDNA evolution in Hawaiian *Drosophila.* These authors found an increased rate of molecular evolution in those lineages that have gone through repeated founder events, as compared to lineages that have diverged primarily due to habitat vicariance.

I tested the hypothesis that rates of mtDNA sequence evolution were constant across sister species' comparisons using the nonparametric relative rate test of Templeton (1983, 1986). This test is dependent on a phylogenetic estimate, and if one is interested in determining how sequence differences are allocated on a branch separating sister species, the test also requires an outgroup sequence to root the tree. This can be problematic for at least two reasons. First, root placement is often uncertain, particularly when outgroup sequences are divergent with respect to ingroup sequences (Maddison, Ruvolo, and Swofford 1992; Templeton 1993; Castelloe and Templeton 1994). It is therefore necessary to consider alternative, statistically indistinguishable root placements in statistical tests. Second, even for a single topology, character states in the outgroup may be polymorphic or unique with respect to the ingroup, creating ambiguities in character state reconstruction.

As above, I estimated haplotype relationships using maximum parsimony, but included outgroup sequences. For a given MP topology (or topologies), nucleotide substitutional changes were reconstructed assuming "Fitch" parsimony. In comparing sister species, the number of unambiguous mutational changes reconstructed per branch (using the TRACE ALL CHANGES facility of MacClade 3.0, Maddison and Maddison 1992) , from a common ancestral node, was summed for each contrast. Each mutational change was given a score. In this analysis, transversional changes were given a score two times that of transitional changes, reflecting the approximate 2:1 transition : transversion ratio observed in comparing sequences from populations of sister species. To test for rate constancy between populations, the difference between their scores was calculated, the resulting sign scores were ranked, and a two-tailed Wilcoxon matched-pairs signed-ranks test was used to convert the results into a statistical statement (Templeton 1983; 1986).

Results

Mitochondrial DNA sequences were gathered for a total of 256 individuals from 25 *Nesticus* populations (table 1). Sample sizes within populations ranged from 6 to 12 individuals, with an average sample size per population of approximately 10 spiders. All individuals were scored for sequence variation at 387 nucleotide sites, corresponding to 129 codons of the ND1 gene. Aligned sequences are available on request from the author.

DNA Polymorphism

DNA polymorphism statistics are summarized in table 2. Considering the entire array of populations, eight populations were fixed for a single haplotype. The

maximum number of haplotypes found within any single population was four. These haplotypes are invariably closely related, differing by at most three mutational differences. About two thirds of the segregating sites within populations involve silent substitutions at third-position codon sites (25 of 36 total mutations).

Estimates of DNA polymorphism based on the number of segregating nucleotide sites per sequence $\theta(S)$ and the average number of nucleotide differences per sequence between all pairs of sequences $\theta(\bar{k})$ are comparable in any given population, reflected by nonsignificant D statistics (table 2). Two exceptions include the Starne's and Walkthrough cave populations of *N. tennesseensis*. In both populations, Tajima's D is significantly negative, indicating an excess of unique or low-frequency polymorphism consistent with directional selection. However, as noted above, nonequilibrium demographic conditions (e.g., fluctuations in population size) can also result in D values significantly different from zero, leading to a false rejection of the null hypothesis (Tajima 1989*a*). For a single marker study such as this, these factors are confounded.

If we assume that genetic drift and mutation are the primary agents determining DNA polymorphism levels, and that mutation rates are constant, then relative levels of sequence polymorphism should reflect variation in population size among populations. There are no striking trends in levels of DNA polymorphism across populations. Although the most genetically diverse population (Grandview State Park) is found on the "surface," there are both troglophilic (e.g., Cassell's Farm, Norris Dam, and Indian caves) and deep-cave populations (e.g., Burton's cave) that show comparable levels of diversity. Species pair comparisons are no more revealing. For example, populations of the troglobitic species *N. holsingeri* are generally more diverse than those of the troglophilic *N. novspl*, but this difference is nonsignificant if one considers the high variance associated with the diversity estimates (table 2). The most biologically significant comparison may involve the relatively polymorphic populations of *N. tennesseensis* versus the single known population of *N. dilutus*, which is monomorphic.

Population Subdivision and Divergence

The observed numbers and proportions of nucleotide differences between populations are given in table 3. These divergence values are uncorrected for multiple hits, and are likely to underestimate divergence for more distantly related sequences. Furthermore, because these divergence values are based on a nonrandom sample of short sequences, they represent biased estimates and therefore should be viewed with caution (Martin, Kessing, and Palumbi 1990). For all population comparisons, the average number of nucleotide differences between haplotypes within populations was substantially lower than that observed between populations (table 3). Correspondingly, pairwise F_{ST} estimates formulated as the fraction of the total mtDNA diversity apportioned among populations were high, ranging from a minimum of 0.884 to a theoretical maximum of 1.0.

Mitochondrial sequences from the type population of *N. carolinensis* are more similar to sequences from either population of *N. mimus* than the *N. mimus* sequences are to each other. Because parsimony analyses also suggest this relationship (i.e., that *N. mimus* is either poly-/or paraphyletic with respect to *N. carolinensis*), I treat these taxa together in the remainder of analyses presented. Within *N. paynei*, the observed proportion of nucleotide differences ranges from 1.7% to 4.4%, with relatively high divergences observed even for geographically adjacent populations (e.g., separated by less than 10 km; fig. 1). For example, haplotypes from Roaring Springs and Coppock's cave differ by an average of 6.18 nucleotide substitutions. Haplotypes from Sensabaugh Saltpeter and Wolfe caves, separated by Clinch Mountain, show 10 fixed substitutional differences.

Within the troglobitic *N. holsingeri*, haplotypes from Burton's cave differ by about 5% from those of Alley and Pond caves. Alley and Pond caves are geographically close, situated within the same karst system (Rye Cove) less than 5 km apart (fig. 1). Even so, sequences from these different populations exhibit fixed differences at three sites (table 3). Maximum divergence values (5.2%) across populations within the troglophilic *N. novspl* are comparable to those within *N. holsingeri*.

Divergence values between *N. tennesseensis* populations are high, ranging from 2.4% up to 7%. Populations from Fallen Rock and Cassell's Farm caves are particularly divergent, exceeding many of the comparisons between the type population of *N. dilutus* (Grassy Creek cave) and *N. tennesseensis*. The divergence values observed between populations of this species pair (4.8%–8.2%) are less than those observed between *N. paynei* and *N. mimus/N. carolinensis* populations (9.4%–11.6%) or *N. novspl* and *N. holsingeri* populations (11.1%–13%).

Genealogical Analyses

Maximum-parsimony analyses of sequence data for the species *N. paynei*, *N. mimus*, and *N. carolinensis* were based on a matrix including 64 variable sites, 54 of which were potentially parsimony-informative. The proportion of sites variable at different codon positions was 13, 1, and 50 for first, second, and third codon positions, respectively. Tree searches of 18 haplotypes for these species result in a single most-parsimonious tree (fig. 2*A*). The most evident characteristic of the MP topology is population monophyly. That is, intrapopulational coalescent events always occur prior to (looking backwards in time) interpopulational coalescent events, resulting in complete correspondence between geographic location and genealogical structure at the population level. Relationships between populations within each species are not strictly concordant with geography and/or prior taxonomy. For example, within *N. paynei*, haplotypes sampled from Norris Dam cave are genealogically distant from Coppock's and Roaring Springs cave haplotypes (fig. 2*A*), even though these populations are geographically close. As discussed above, mtDNA haplotypes of *N. mimus* are not monophyletic with respect to those of *N. carolinensis*.

Table 3
Within- and Between-Population Sequence Divergence Values

N. tennesseensis AND *N. dilutus*

	IC	GV	RC	FR	CF	BC	SC	WT	GC
IC.....	**1.545** **(1.545)**	18.85 (15.85)	19.5 (15.5)	23.23 (19.23)	19.7 (16.7)	18.83 (15.56)	16.29 (13.19)	21.13 (19.13)	28.33 (23.33)
GV	0.049	**1.694** **(1.694)**	14.61 (11.61)	25.35 (20.35)	21.48 (19.48)	9.48 (7.20)	10 (7.90)	11.44 (10.44)	19.78 (18.78)
RC.....	0.050	0.038	**0.303** **(0.303)**	26.74 (20.74)	20.53 (17.53)	16.83 (13.56)	12.93 (9.83)	15.63 (13.63)	19.83 (14.83)
FR.....	0.060	0.066	0.069	**0.509** **(0.509)**	15.81 (12.81)	23.18 (17.91)	23.91 (18.81)	26.71 (22.71)	31.91 (24.91)
CF.....	0.051	0.057	0.053	0.041	**1.178** **(1.178)**	19.43 (17.16)	19.7 (17.6)	20.5 (19.5)	23.7 (19.7)
BC.....	0.049	0.024	0.043	0.060	0.050	**0.873** **(0.436)**	10.1 (9.73)	11.55 (10.28)	22 (17.73)
SC.....	0.042	0.026	0.033	0.062	0.051	0.026	**0.200** **(0.0)**	12.9 (11.8)	20.1 (16)
WT....	0.055	0.030	0.036	0.070	0.053	0.030	0.033	**0.327** **(0.327)**	18.8 (15.8)
GC	0.073	0.051	0.051	0.082	0.061	0.057	0.052	0.048	**0**

N. novsp1 and *N. holsingeri*

	ST	NS	CS	AC	PC	BU
ST.....	**0**	20 (16)	18 (14)	49.1 (35.1)	49.18 (36.08)	42.83 (29.83)
NS.....	0.052	**0**	9 (3)	50.1 (34.1)	50.18 (35.08)	45.83 (30.83)
CS.....	0.046	0.023	**0.360** **(0.360)**	49.1 (31.1)	49.18 (32.08)	44.83 (27.83)
AC.....	0.127	0.129	0.127	**0.180** **(0.180)**	3.28 (0.18)	20.28 (17.28)
PC.....	0.127	0.130	0.127	0.008	**0.360** **(0.180)**	19.36 (17.26)
BU.....	0.111	0.118	0.116	0.052	0.050	**1.0** **(1.0)**

N. mimus/N. carolinensis AND *N. paynei*

	GM	LG	LC	CC	GS	ND	RS	CV	SS	WC
GM...	**1.054** **(1.054)**	11.73 (7.73)	7.39 (6.21)	36.12 (23.12)	37.45 (27.62)	42.91 (31.91)	35.45 (23.45)	39.45 (29.45)	37.45 (25.45)	37.45 (26.45)
LG ...	0.032	**0**	6.36 (3.18)	35.67 (20.67)	37 (25.17)	39 (26)	35 (21)	37 (25)	37 (23)	37 (24)
LC ...	0.019	0.017	**0.654** **(0.327)**	35.03 (22.85)	36.36 (27.35)	40.36 (30.18)	34.36 (23.18)	38.36 (29.18)	36.36 (25.18)	36.36 (26.18)
CC ...	0.098	0.097	0.095	**0.500** **(0.500)**	11.67 (6.83)	16.52 (12.52)	6.67 (5.67)	15.67 (12.67)	9.67 (4.67)	14.67 (10.67)
GS ...	0.102	0.101	0.099	0.032	**0.667** **(0.333)**	11.85 (9.02)	11 (7.17)	9.67 (7.83)	8 (4.17)	11.33 (9.5)
ND ...	0.116	0.106	0.109	0.042	0.032	**1.2** **(1.2)**	16.27 (13.27)	6.18 (5.18)	13.27 (10.27)	18.27 (16.27)
RS ...	0.096	0.095	0.094	0.018	0.030	0.044	**0**	15 (13)	9 (5)	14 (11)
CV ...	0.107	0.101	0.104	0.043	0.026	0.017	0.041	**0**	10 (8)	15 (14)
SS....	0.102	0.101	0.099	0.026	0.022	0.036	0.024	0.027	**0**	10 (7)
WC...	0.102	0.101	0.099	0.040	0.031	0.050	0.038	0.041	0.027	**0**

NOTE.–Above diagonal: the average number of nucleotide differences between sequences from populations X and Y. Diagonal (in bold type): the average number of nucleotide differences between all pairs of sequences within populations ($\hat{k}$). Values on and above the diagonal are based on either the observed number of total site differences or the observed number of transitional site differences (in parentheses). Below diagonal: the average proportion of nucleotide differences between sequences from popultions X and Y. All divergence values are uncorrected for multiple hits. Population acronyms are as given in table 1.

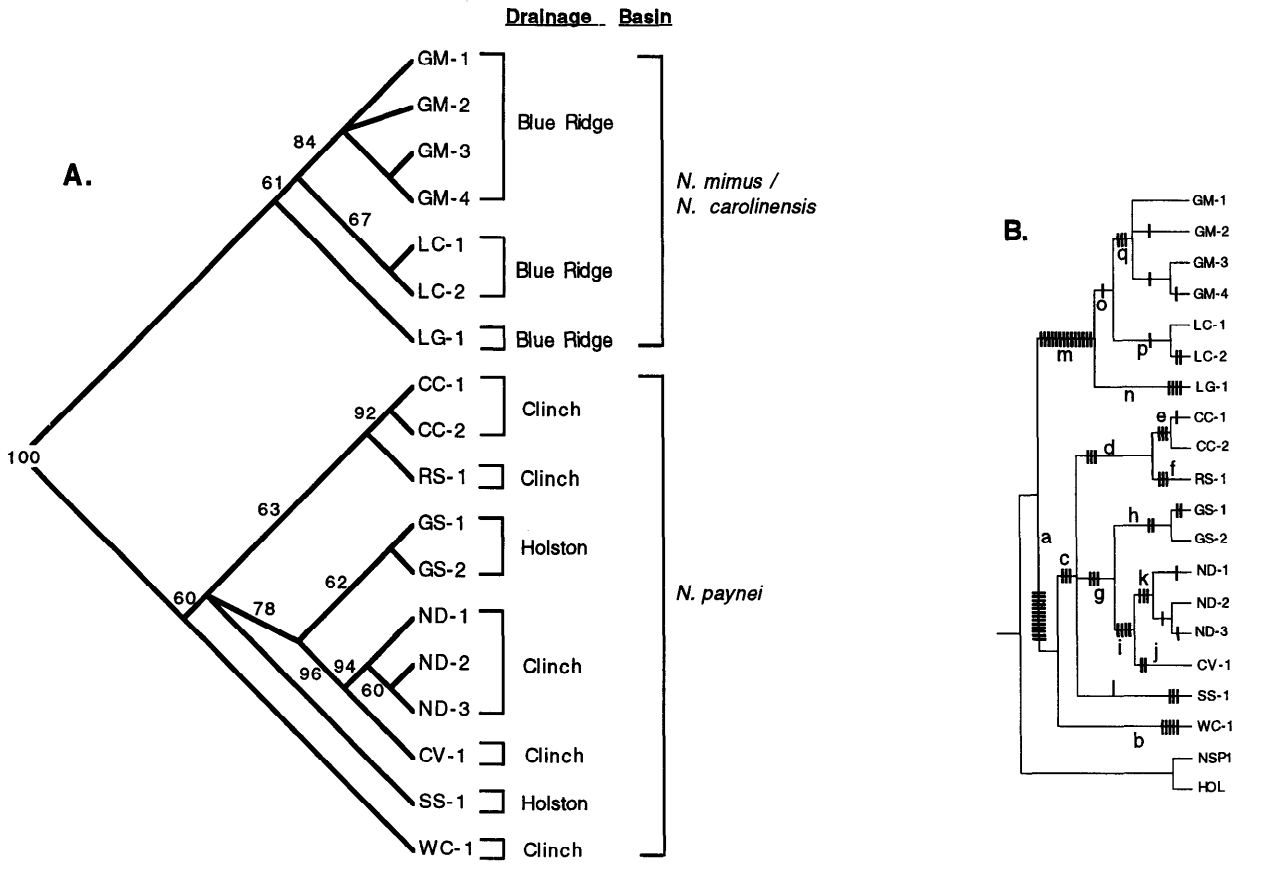

FIG. 2.—*A*, Maximum-parsimony results for the species *N. paynei, N. carolinensis,* and *N. mimus.* The tree shown is a single most-parsimonious tree resulting from a branch-and-bound search of 18 mtDNA haplotypes. Tree length = 90, and ensemble consistency index (CI) = 0.797, excluding uninformative characters. Zero-length branches were collapsed in tree searches. Bootstrap values, based on 100 replicates, are shown along branches. Tree skewness statistics, calculated using PAUP (Swofford 1993) from 10,000 random trees, indicate significant phylogenetic information for these data ($g1 = -0.5852$ for 18 terminals and 64 variable sites, $P < 0.01$ from table 2 of Hillis and Huelsenbeck 1992). *B,* Tree topology and mutational allocations used in test of the rate constancy hypothesis for haplotypes of *N. paynei, N. carolinensis,* and *N. mimus.* Maximum-parsimony tree topology was estimated using *N. holsingeri* and *N. novspl* haplotypes as outgroups. Each branch or branch segment is designated with a lowercase letter, with optimization of mutational changes along branches. Only those changes which are unambiguous under parsimony are shown; the majority of parsimony-ambiguous reconstructions are associated with the branch separating *N. paynei* from *N. carolinensis/N. mimus,* reflecting uncertain/polymorphic character states in the outgroup.

For the species pair *N. holsingeri* and *N. novspl,* branch-and-bound analyses of eleven haplotypes resulted in a single most-parsimonious tree (fig. 3*A*). The proportion of sites variable for these data are 13, 3, and 56 for first, second and third codon positions, respectively. Similar to the results for *N. paynei* and *N. carolinensis/N. mimus,* there is complete correspondence between geographic location and genealogical structure at the population level. Genealogical relationship is also associated with geography above the population level, where geographically close populations also form monophyletic clades. These relationships are supported by high bootstrap proportion values, reflecting few homoplastic sites and a relatively high character : terminal ratio (55 parsimony-informative sites: 11 terminals).

Finally, parsimony analyses of 24 *N. tennesseensis* and *N. dilutus* haplotypes resulted in a single most-parsimonious tree (fig. 4*A*). This result was based on a matrix including 62 variable sites, 49 of which were potentially parsimony-informative. The proportion of sites variable at first, second, and third codon positions was 16, 2, and 44, respectively. Population monophyly also obtains, for haplotypes of *N. tennesseensis* and *N. dilutus,* a conclusion strongly supported by bootstrap resampling (fig. 4*A*). Genealogical relationships between haplotypes of different populations are inconsistent with simple geographical expectations. However, many of the character changes supporting relationships between populations are ultimately homoplastic, resulting in internal branches with low bootstrap values. This high level of homoplasy is not surprising considering that, relative to the above comparisons, the *N. tennesseensis/N. dilutus* comparisons involve a combination of more terminal taxa but fewer parsimony-informative sites.

Because of the complete correspondence between geographic location and genealogical structure at the population level, I used Slatkin's (1989) coalescent approach to estimate (N_m). The aim of this approach is to assess and place an upper limit on the probability that all n sequences drawn from a population are descended from an ancestral sequence that was in that population, given some arbitrary level of gene flow. Slatkin (1989) termed this the "probability of non-immigrant ancestry" or $P(n, N_m)$. If $(N_m) > N_{mcrit}$, where $N_{mcrit} = 0.05$, then

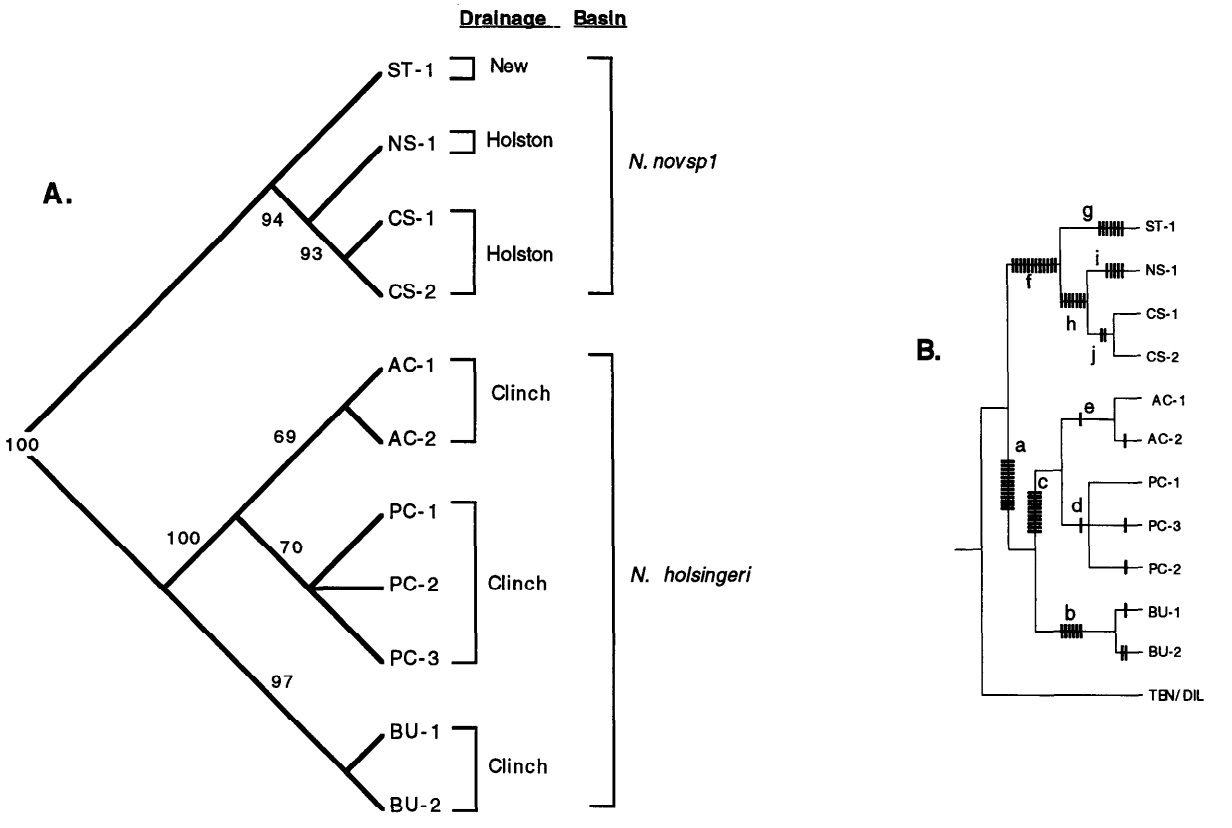

FIG. 3.—*A*, Maximum-parsimony results for the species pair *N. holsingeri* and *N. novsp1*. The tree shown is a single most-parsimonious tree resulting from a branch-and-bound search of 11 mtDNA haplotypes. Tree length = 81, ensemble CI = 0.952. Bootstrap values are shown along branches. Tree skewness statistics indicate significant phylogenetic information for these data (g1 = −0.8567 for 11 taxa and 72 variable sites, $P < 0.01$). *B*, Tree topology and mutational allocations used in test of the rate constancy hypothesis for haplotypes of *N. holsingeri* and *N. novsp1*. Maximum-parsimony tree topology was estimated using *N. tennesseensis* and *N. dilutus* haplotypes as outgroups. Mutational changes along branches were optimized.

the probability of observed "non-immigrant ancestry" is less than 5%. For the *Nesticus* data, given 6–12 sequences sampled per population, and the observation of population monophyly for all populations, N_{mcrit} values range from approximately 0.6 to 1.0. These estimates are conservative, treating each population as independent. If one could show that the probability of gene exchange between populations is somehow related to some variable (e.g., geographic location), then $P(n, N_m)$ values would more appropriately be calculated as joint probabilities, decreasing N_{mcrit} values (Slatkin 1989).

Relative Rates

Maximum-parsimony tree topologies, with reconstructed substitutional changes, are shown in figures 2*B*–4*B*. Sequences from the most closely related outgroup taxa were used to polarize parsimony trees for each species pair. For *N. paynei–N. carolinensis/N. mimus* and *N. holsingeri–N. novsp1*, parsimony placed the root unequivocally on the basal branch separating species. Root placement for the species *N. tennesseensis* and *N. dilutus* was ambiguous, with equally parsimonious reconstructions placing the root either on the branch separating *N. dilutus* from *N. tennesseensis* or on the branch separating the Rumbold's cave population of *N. tennesseensis* from the remainder of the populations (fig. 4*B*). Statistical tests were performed using both root placements. The substitutional data used in the Wilcoxon matched-pairs signed-ranks test of the rate equality hypothesis are shown in table 4, with results of these tests for each species pair summarized in table 5. On the basis of available data there is no evidence for mtDNA rate inequality.

Discussion

Population Genetic Structure and Sampling

There are two related patterns that concisely summarize the genetic structure of mtDNA variation in the *Nesticus* species sampled. First, multiple individuals sampled from the same population always share mtDNA sequences that are either identical or mutationally closely related. Genealogically, such haplotypes are *always* exclusive with respect to sampled haplotypes from other populations. Second, haplotypes from different populations are mutationally divergent, and to find a most recent common ancestor for haplotypes from different populations requires us to consider relatively long branches (long with respect to those within populations).

These general patterns of sequence monophyly within populations, with large divergences between populations, are consistent with those expected under a model of population fragmentation through habitat vicariance (Larson, Wake, and Yanev 1984; Templeton et

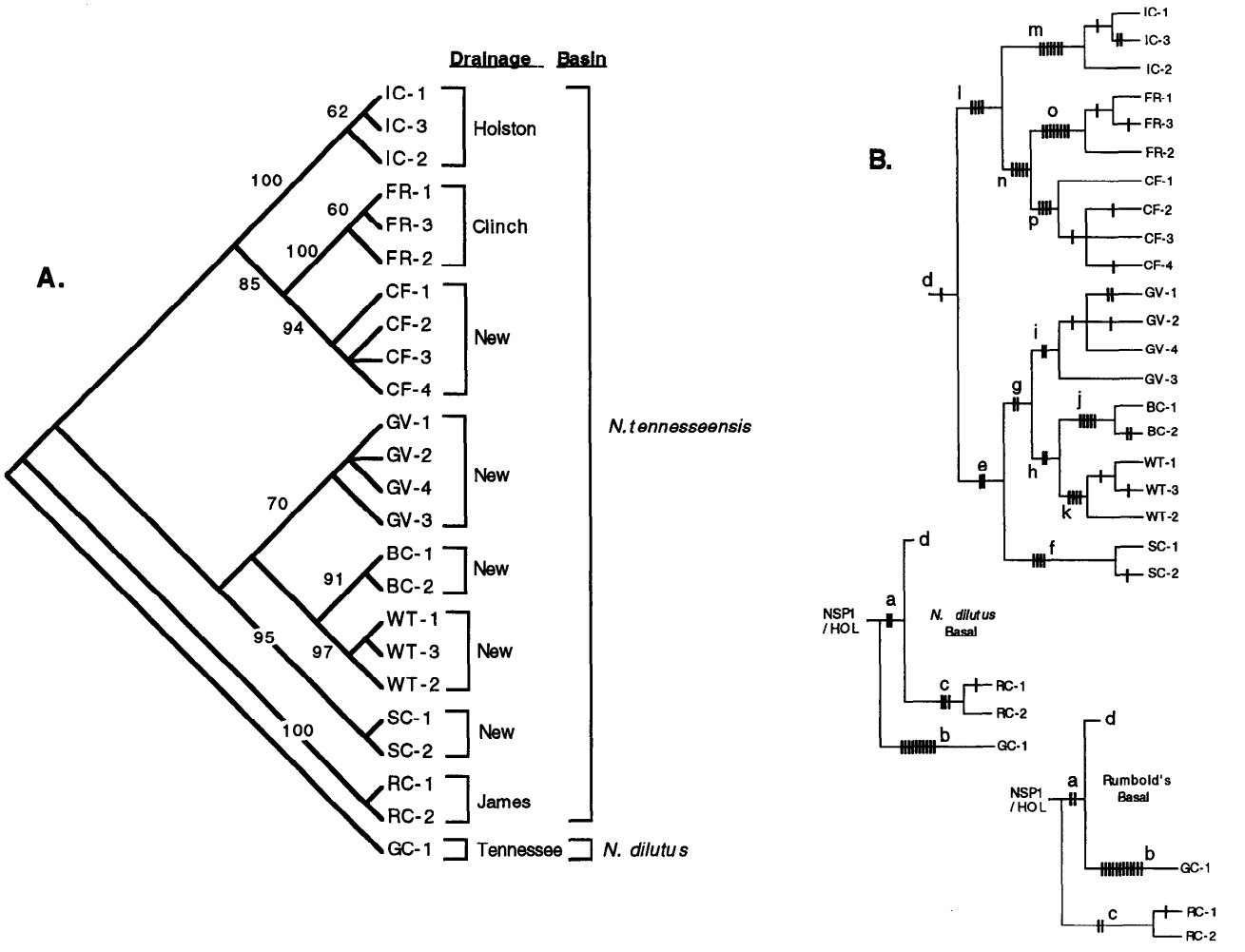

FIG. 4.—*A*, Maximum-parsimony results for the species-pair *N. tennesseensis* and *N. dilutus*. The tree shown is a single most-parsimonious tree resulting from a branch-and-bound search of 24 mtDNA haplotypes. Tree length = 93, ensemble CI = 0.675. Bootstrap values are shown along branches. Tree skewness statistics indicate significant phylogenetic information for these data (g1 = −0.7152 for 24 taxa and 62 variable sites, $P < 0.01$). *B*, Tree topology and mutational allocations used in test of the rate constancy hypothesis for haplotypes of *N. tennesseensis* and *N. dilutus*. Maximum-parsimony tree topologies were estimated using *N. holsingeri* and *N. novspl* haplotypes as outgroups. Root position is uncertain, with either *N. dilutus* (GC1) or Rumbold's Cave (RC) haplotypes as basal. Mutational changes along branches were optimized incorporating uncertainly in root placement.

al. 1990; Gerber 1994). Under such a model, an interbreeding population is fragmented into several habitat-limited subpopulations at some time in the past, and, subsequent to fragmentation, the exchange of migrants among these subpopulations is extremely restricted to nonexistent. As such, the genetic structure of such a species is largely a result of historical, rather than recurrent, processes.

However, inferences about population genetic structure are ultimately contingent on sampling, including both within-population sampling and the geographic scale of population sampling (e.g., Templeton 1993; Jackman and Wake 1994; Templeton, Routman, and Phillips 1995). For example, if samples are taken for relatively few individuals from geographically widespread populations, one might necessarily conclude that populations are completely isolated. Alternatively, if one were to increase the number of sequences sampled per population and correspondingly decrease the geographic scale of sampling between populations, conclusions about population structure may differ. For these reasons it is important to consider whether *Nesticus* population samples are sufficient with respect to the evolutionary inferences made above.

The numbers of mtDNA sequences sampled per *Nesticus* population were essentially equivalent across all populations (table 1). Slatkin has shown (assuming a single population, island model) that the probability of population monophyly is less than 5% for a sample of 10 sequences, assuming "moderate" levels of gene flow ($N_m = 1$). For "low" levels of gene flow ($N_m = 0.01$) this probability increases to >95% for equivalent sample sizes (Slatkin 1989, fig. 1). In this respect, sample sizes of 10 sequences, in combination with the observation that all sampled haplotypes are monophyletic at the population level, are sufficient to discriminate low from moderate levels of gene flow in *Nesticus*.

I have not sampled extensively at the microgeographic level (i.e., samples from populations across an array of geographically adjacent populations). At this

Table 4
Summation of Mutational Changes Used in Wilcoxon Matched-Pairs Signed-Ranks Test of the Rate Equality Hypothesis

N. CAROLINENSIS/ N. MIMUS–N. PAYNEI			*N. NOVSP1– N. HOLSINGERI*			*N. TENNESSEENSIS– N. DILUTUS*		
Branch	TR	TV	Branch	TR	TV	Branch	TR	TV
a	11	2	a	8	5	a^1	2	0
b	4	1	b	5	1	a^2	1	1
c	3	0	c	11	0	b^1	7	3
d	2	1	d	0	1	b^2	9	3
e	2	1	e	0	1	c^1	2	1
f	3	0	f	8	4	c^2	2	0
g	3	0	g	7	0	d	1	0
h	1	1	h	7	0	e	2	0
i	4	0	i	2	3	f	3	1
j	2	0	j	0	2	g	2	0
k	2	1				h	2	0
l	1	2				i	1	1
m	9	5				j	4	1
n	2	2				k	4	0
o	1	0				l	4	0
p	1	0				m	6	1
q	2	1				n	5	0
						o	6	2
						p	4	0

NOTE.—For each species pair, the number of transitional (TR) and transversional (TV) changes are given per branch; branch designations correspond to those in figures 2*B*–4*B*, for the species pairs *N. paynei–N. carolinensis/N. mimus*, *N. novsp1–N. holsingeri*, and *N. tennesseensis–N. dilutus*, respectively. Within-population polymorphisms are not included in these totals. For the *N. tennesseensis–N. dilutus* comparisons, unequivocal optimization of mutational changes on branches a–c differ depending on root placement, with either *N. dilutus* (GC1)[1] or Rumbold's Cave (RC)[2] as basal.

Table 5
Wilcoxon Matched-Pairs Signed-Ranks Test of the Rate Equality Hypothesis

Comparisons	Smallest Signed Rank	*N*
N. dilutus and *N. tennesseensis*		
RC (a, c)–GC (b)	37.5 (34)	15 (16)
IC (a, d, l, m)–GC (b)	141 (171)	24 (26)
FR (a, d, l, n, o)–GC (b)	172 (216)	30 (32)
CF (a, d, l, n, p)–GC (b)	159 (198)	26 (28)
GV (a, d, e, g, i)–GC (b)	73.5 (91.5)	19 (21)
BC (a, d, e, g, h, j) GC (b)	109.5 (121.5)	21 (23)
WT (a, d, e, g, h, k)–GC (b)	136.5 (149.5)	23 (25)
SC (a, d, e, f)–GC (b)	73.5 (91.5)	19 (21)
N. paynei and *N. carolinensis/N. mimus*		
LC (m, o, p)–WC (a, b)	294	34
LC (m, o, p)–ND (a, c, g, i, k)	385	42
LG (m, n)–WC (a, b)	297	36
LG (m, n)–ND (a, c, g, i, k)	469	44
N. novsp1 and *N. holsingeri*		
ST (f, g)–AC (a, c, e)	420.5	44
ST (f, g)–PC (a, c, d)	420.5	44
ST (f, g)–BU (a, b)	347.5	38
NS (f, h, i)–AC (a, c, e)	609.5	49
NS (f, h, i)–PC (a, c, d)	609.5	49
NS (f, h, i)–BU (a, b)	423.5	43
CS (f, h, i)–AC (a, c, e)	505.5	46
CD (f, h, i)–PC (a, c, d)	505.5	46
CS (f, h, i)–BU (a, b)	395.5	40

NOTE.—The data used to generate the comparisons come from the summation of mutational changes along branch segments as given in table 4. For the species pair *N. paynei* and *N. carolinensis/N. mimus*, rankings are given for a range of pairwise comparisons between species (representing extremes in branch lengths). All other rankings are given for all pairwise comparisons between species. For the species pair *N. dilutus* and *N. tennesseensis*, comparisons reflect the uncertainty in root placement, with values calculated assuming either *N. dilutus* or Rumbold's Cave (value in parentheses) as basal. Probability values for getting signed-rank values this small or smaller, as a function of *N* (the number of nonzero ranks), are all $P > 0.05$, using a two-tailed test (Rohlf and Sokal 1981, table 30).

level, I expect there to be "unsampled" populations geographically adjacent to any given sampled population. The existence of unknown and unsampled populations is almost certain, given the cryptic nature of *Nesticus* spiders in combination with the thousands of potentially suitable habitats available. For example, Holsinger and Culver (1985) cite a minimum of 2,611 known caves in the Appalachian Valley and Ridge of northeastern Tennessee and southwestern Virginia. How might the existence of unsampled populations influence my conclusions about restricted gene flow? If such unsampled populations are exchanging migrants with sampled populations at moderate to high levels, this should be evidenced as higher levels of within-population diversity (see Hudson 1990, fig. 7). The observation of relatively low and equivalent polymorphism estimates across all sampled populations and the observation that the minimal between-population divergence value (0.008 between Alley and Pond cave populations of *N. holsingeri*) exceeds the maximal within-population diversity value (0.0045 within the Grandview SP population) suggest reduced levels of both observed and "unobserved" gene flow.

Population Structure and Ecological Differences

Prior genetic studies of cave-dwelling arthropods have generally revealed variation in population structure corresponding to differences in habitat dependence, given that variation in other factors is controlled for (e.g., geographic scale of sampling, area geology, population history). For example, Caccone (1985) estimated N_m in 11 species of terrestrial cave-dwellers using both quantitative and qualitative methods (Slatkin 1985), and concluded that moderate to high rates of gene flow characterize troglophilic and epigean species. Troglobitic species, on the other hand, were characterized as having restricted gene flow. These predicted differences do not hold for Appalachian *Nesticus*, with species characterized by similar and essentially complete population subdivision regardless of differences in habitat dependence. There are at least three explanations for this discrepancy.

First, I used variation in mtDNA sequences to estimate population structure, whereas the majority of previous studies have used protein electrophoresis data. Assuming that the sexes are demographically equivalent, the maternal haploid inheritance of mtDNA reduces the effective amount of gene flow by a factor of four with respect to a diploid nuclear genetic system (Birky, Muruyama, and Fuerst 1983). In this respect, Appalachian *Nesticus* may be much less structured at nuclear loci, and this would explain the discrepancy between estimates of population genetic structure in the European

N. eremita (with moderate to high levels of gene flow, Caccone 1985) and those of this study. Furthermore, if dispersal rates are male-biased and variable across ecological categories in *Nesticus,* then different species might show differing levels of genetic structuring at nuclear loci.

Second, previous studies may have underestimated population subdivision because of shared ancestral polymorphism. For example, under a fragmentation model, populations that currently exchange no genes can still share allozyme polymorphism, erroneously implying gene flow (Larson, Wake, and Yanev 1984; Slatkin 1985; Templeton, Routman, and Phillips 1995). In fact, Caccone (1985) commented that dispersal ability did not correlate well with apparent levels of gene flow in some instances, where species with known limited dispersal ability had moderate to high levels of measured gene flow.

Finally, my use of "habitat preference" as a surrogate for potential differences in population biology may be inappropriate. In accord with previous studies, I predicted that a combination of (1) more continuous suitable habitat (e.g., montane "surface" microenvironments), (2) increased gene flow opportunity either through ballooning or dispersal through "microcavernous" habitats (see Holsinger and Culver 1985) , and/or (3) larger population sizes would result in less population structuring in species occupying habitats near or on the "surface."

The data presented here and in previous analyses are inconsistent with this prediction. Even though I have only included a single epigean species in this study (*N. mimus*), surveys of intraspecific mtDNA divergence in other high-elevation epigean *Nesticus* reveal high levels of genetic differentiation across geographically close populations (Hedin 1995). In addition, epigean species of *Nesticus* are typically locally distributed, and several such species are known only from single localities (Gertsch 1984; Hedin 1995). Finally, despite focused search efforts, many of my collections consist of less than 10 individuals per locality, and many of the original descriptions of epigean species are based on one to few specimens (Gertsch 1984). Taken together, it seems that limited dispersal capabilities combined with narrow physiological tolerances result in discontinuous population structures and relatively small population sizes in both cave *and* epigean *Nesticus.*

Species and Speciation

Throughout this paper I have treated *Nesticus* species as morphologically defined lineages, or groups of populations which share both history and genital morphologies. I have argued above that the exchange of migrants among populations within such lineages is currently extremely restricted to nonexistent, as a result of both extrinsic (e.g., complex area geology) and intrinsic (e.g., narrow physiological limits) properties. This fragmentation has resulted in groups of discrete, perhaps independent, populations; the large sequence divergences observed between populations suggest that this independence may extend well into the past (Hudson 1990; Hey 1991). I discuss below how these patterns of mtDNA genealogical structure and divergence potentially relate to questions about species and processes of speciation, including vicariance and founder event speciation.

Phylogenetic data indicate that *Nesticus* spiders from the same population always share a unique combination of characters, which provides evidence for divergence from other populations (see Nixon and Wheeler 1990). The genetic data also suggest that breeding populations are geographically localized: for example, the individuals interacting within the confines a single cave or boulder field. Finally, the mtDNA divergence values observed between morphologically similar populations are high (ranging up to 7%; table 3), generally exceeding both the corresponding intraspecific values of up to 5% reported for beetles and *Drosophila* (see Vogler et al. 1993, fig. 1), and interspecific values reported for 12S mtDNA sequences of Hawaiian *Tetragnatha* spiders (Gillespie, Croom, and Palumbi 1994). Taken together, these patterns imply that species as morphological lineages are currently more inclusive than the basal evolutionary or phylogenetic units, both of which correspond to local populations. Such patterns are perhaps indicative of incipient or cryptic speciation.

However, there are two reasons for retaining the category "species" for lineages of morphologically similar populations, both of which require that we regard observed patterns of mtDNA structure in the context of evolutionary time. First, the observed patterns of mtDNA structure in *Nesticus,* and the basal evolutionary or phylogenetic units based on such patterns, might potentially be ephemeral over geologic or evolutionary time (de Queiroz and Donoghue 1988; Avise and Ball 1990; Frost and Hillis 1990). As noted above, the phylogeographic distribution of mtDNA lineages reflects only female migration and effective population size; genetic structure at nuclear loci is conceivably less extreme. Related to this argument is the expected rapid rate of mtDNA lineage sorting in isolated populations. 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 (see also Avise and Ball 1990, fig. 2). These times decrease as female effective population sizes decrease.

Biogeographic considerations are consistent with this notion of ephemeral units. For example, glacial–interglacial episodes of the Pleistocene Epoch are hypothesized to have strongly influenced cave-dwelling lineages of the southern Appalachians (summarized in Barr 1985; Holsinger and Culver 1985; Holsinger 1988), with periods favorable for population exchange (e.g., glacials) separated by conditions promoting population fragmentation (e.g., interglacial maxima). If this hypothesis obtains for *Nesticus,* then any single climatic extreme is only one in a series of similar, regularly recurrent extremes, and species fragmentation in *Nesticus* might be viewed as an evanescent, yet recurring, phenomenon in "species time" (see also Vrba 1995).

Viewing species as morphological lineages does not come without problems. Unless we can predict the future, we cannot know whether present-day separations are temporary or permanent. For instance, the disjunct Burton's cave population of *N. holsingeri* is not likely to ever interact with other *N. holsingeri* populations in the future. The only avenue of dispersal for these troglobitic spiders is subterranean, which is impossible for geologic reasons; future climatic fluctuations seem unlikely to cause reticulation in this species. In addition, we are largely ignorant of the evolutionary significance of continuity and discontinuity in *Nesticus* genital morphologies. Although Huber (1993) has suggested that *Nesticus* genitalia have importance in mate recognition, their role in reproductive isolation is unknown. Despite these difficulties, I have opted for the conservative approach; in the discussion that follows I regard species as morphological lineages which are hypothesized to be interacting through evolutionary time (see Frost and Hillis 1990).

Vicariance Speciation

There is little doubt that fragmentation through habitat vicariance has played an important role in the history of *Nesticus* population divergence. Below, I examine specific phylogeographic patterns which underly this divergence, and ask whether we can extrapolate these patterns to the splitting of morphological lineages through habitat vicariance. With the exception of *N. dilutus* and *N. carolinensis/N. mimus,* the species included in this study are distributed over four major drainage basins within the Appalachian Valley and Ridge of southwestern Virginia and northeastern Tennessee. These include the James, New, Holston, and Clinch river drainage basins (table 1). These drainage basins are well defined geographically and contain topographically confined karst areas (Holsinger and Culver 1985). Furthermore, each of these drainage basins has a unique assemblage of endemic terrestrial cave-dwelling taxa (Holsinger and Culver 1985) , thus corresponding to "areas of endemism" (Nelson and Platnick 1981).

A simple vicariant phylogenetic hypothesis predicts that populations (or species) within drainage basins will be more closely related to each other than to populations (or species) from separate drainage basins. This prediction is upheld for the species pair *N. holsingeri* and *N. novspl,* where *N. holsingeri* is endemic to the Clinch river basin and *N. novspl* is found in the adjacent Holston and New River basins (fig. 1). Within *N. novspl,* haplotypes from Holston river drainage populations (CS and NS, see table 1 for population acronyms) are more closely related to each other than to haplotypes from the New River drainage (ST) population (fig. 3*A*). The maximum-parsimony tree for *N. tennesseensis* does not support the drainage basin monophyly hypothesis, as populations from the New River drainage basin (including GV, BC, WT, CF, and SC) are not monophyletic (fig. 4*A*). Searching for trees under the constraint that New River drainage haplotypes form a monophyletic clade results in a single tree seven steps longer than the MP tree. This topology is significantly less parsimonious than the MP tree given the data available ($n = 9$, Ts = 5, $P < 0.05$; Rohlf and Sokal 1981, table 30) , as tested using a one-tailed Wilcoxon signed-ranks test (Templeton 1983). However, this result is due mostly to the exclusion of divergent haplotypes of the Fallen Rock cave population, found in the adjacent Clinch river drainage basin (fig. 1). Constraint trees that include New River populations in addition to the Fallen Rock cave population as monophyletic are not significantly less parsimonious than the MP tree ($n = 4$, $P > 0.05$). This result is not surprising given that the drainage divide separating the New and Clinch river drainage basins is rather weakly defined and contains carbonate rock (Holsinger and Culver 1985). Finally, the drainage basin monophyly hypothesis is not most parsimonious for haplotypes of *N. paynei,* in that neither the Holston river drainage haplotypes (GC and SS) nor the Clinch river drainage haplotypes (CC, RS, ND, CV, and WC) form exclusive clades with respect to one another (fig. 2*A*). However, trees in which haplotypes restricted to each drainage basin form monophyletic clades are not significantly less parsimonious than the MP topology ($n = 4$, $P > 0.05$).

Despite a lack of power in distinguishing alternative hypotheses, the data suggest that the biogeographic history of *Nesticus* diversification involves more than simple lineage vicariance. This hypothesis is supported by previous studies of cave-dwelling lineages from the southern Appalachians. Barr (1985) , based on studies of cave beetles (*Pseudanopthalmus*) from southwestern Virginia and adjacent northeastern Tennessee, suggests that the presence or absence of lineages in any given cave or cave system involves an element of chance that he has called the "shotgun effect." The implication is that phylogenetic relationships in these beetles will reflect the disjunct and apparently stochastic patterns of observed geographic distributions, without a close correspondence to the geographical distance between populations, surface topography, or area geology. Taken together, these studies suggest that "pre-fragmentation" processes have played an important role in the speciational history of Appalachian lineages. Consistent with the climatic-fluctuation hypothesis, such processes probably involved a complex pattern of dispersal, vicariance, and competition during geologic periods favorable for population exchange.

Founder Event Speciation

Sampling events involving reductions in population size are often invoked as important processes *inducing* speciation in cave-dwelling lineages, particularly in cave-limited species (see reviews of Sbordoni 1982; Barr and Holsinger 1985). Such arguments are founded on the common premise that reductions in population size characterize cave populations, and that these reductions are often coincident with a shift to the novel selective regime of the cave environment (Howarth 1983; Barr 1985). I make the argument below that founder event modes of speciation, involving the establishment of a new population from one or a few founder individuals (Carson and Templeton 1984), are not likely to have

played an important role in the speciation process of *Nesticus* spiders.

First, I cannot reject the null hypothesis of rate constancy in mtDNA sequences between populations either within species (results not shown) or across species. Given the short sequences available, this is a rather weak test for founder events, and even if I were able to reject rate constancy, I could not attribute rate variation exclusively to population size effects (cf. DeSalle and Templeton's [1988] comparative analyses of mtDNA evolution in Hawaiian *Drosophila*). A more powerful test will involve sequences for multiple loci.

More importantly, theory dictates that a highly discontinuous population genetic structure is not conducive to speciation through founder events (Templeton 1980*b*). In a species subdivided into small populations with restricted gene flow among populations, both the variance and inbreeding effective sizes are predicted to be small, resulting in populations characterized by high individual-level homozygosity and restricted genetic backgrounds (Templeton 1980*b*). Founder populations derived by sampling individuals from within a local population will have little impact on variation in the inbreeding effective size and the genetic environment in which selection takes place. Even if founder individuals comprise a random sample from several ancestral populations (e.g., a pattern potentially consistent with the climatic fluctuation hypothesis), inbreeding in the founders will most likely reestablish the ancestral genetic environment (Templeton 1980*b*).

Under either sampling scenario, the probability for a founder event *inducing* speciation is low, and I argue that this low probability obtains for cave populations of *Nesticus*. This is not to say that sampling events resulting in reductions in population size are unimportant in the speciation history of *Nesticus*, only that such events do not result in "founder event" speciation per se. Instead, I think the primary role of founder events, if they occur, is to establish geographically isolated populations that simply represent additional isolated demes of the ancestor. Processes subsequent to population isolation, rather than properties of population establishment, likely play the more important role in promoting speciation.

Sequence Availability

DNA sequences of the most common haplotype for each of the populations analyzed have been submitted to GenBank, with accession numbers U40499–U40523.

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APPENDIX

Primers Used in mtDNA PCR Amplifications

Sequence comparisons include data for the hexapods *Drosophila* (Clary and Wolstenholme 1985) , *Locusta* (Flook, Rowell, and Gellissen 1995) and *Apis* (Crozier and Crozier 1993) , and the spiders *Hypochilus, Habronattus,* and *Nesticus* (unpublished data). Spider comparisons include representatives of both primitive (*Hypochilus*) and more derived (*Nesticus* and *Habronattus*) spider families. Primers are numbered following Simon et al. (1994).

(N1-J-12261).....	TCRTAAGAAATTATTTGAGC	[20mer]
Drosophila.....	..A.....AG.....	
Locusta.....	..A.....AG.....	
Apis	..AA.T.....GTT.T	
Habronattus.....	..A.....	
Nesticus	..G.....	
(TL1-N-12718)	TGCATTAGAATTAGAATCTA	[20mer]
Drosophila.....	....A..A.T.....T..	
Locusta.....	....A.GA.T.....TC.	
Apis	.....A.T....G..T..	
Habronattus.....	.....	
Nesticus	.....	
Hypochilus.....	...T...A.T.....T..	

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