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MOLECULAR EVOLUTIONARY DIVERGENCE AMONG NORTH AMERICAN CAVE CRICKETS. I. ALLOZYME VARIATION

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Abstract.—Forty-nine populations of nine species of North American cave crickets (genera *Euhadenoecus* and *Hadenoecus*) have been studied for genetic variation at 41 loci by electrophoresis. Wright's F_{ST}, Slatkin's Nm^* gene-flow estimator, and Nei's genetic distances (D) have been used to compare closely related species that have different ecological requirements (cave vs. forest species), distribution patterns, and/or different degrees of geographic isolation among populations.

Cave and epigean (noncave) species differ greatly in their levels of genetic differentiation. Cave species have lower rates of gene exchange (low Nm, high D, and F_{ST}) than epigean species. Within cave species the degree of genetic differentiation among populations is correlated with the limestone structure of the area where the species occur. Species or groups of populations inhabiting areas where the limestone is continuous and highly fissured (e.g., *H. subterraneus* populations in the Mammoth Cave region) are genetically less differentiated than are populations occurring in regions where the limestone distribution is more fragmented, such as the Appalachian Ridge where *E. fragilis* occurs; this effect is more extreme in Central Tennessee where genetically differentiated *E. insolitus* populations occur only a few kilometers apart. This suggests that epigean dispersal through forest habitat in cave-dwelling species is negligable. For forest species, the data indicate relatively recent radiation with ongoing gene exchange among populations. For cave species, the distribution of protein polymorphisms is apparently more a function of historical patterns of gene exchange rather than current gene exchange.

Phylogenetic relationships were studied using cluster analyses (UPGMA and Wagner algorithms) of Nei's and Edwards' genetic distances and multivariate analysis (correspondence analysis) of the raw allele frequencies. Different algorithms result in branching patterns that are similar but not entirely concordant with one another or with the phylogeny based on morphology.

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Population genetics theory has long emphasized the importance of genetic structure of species, that is, the distribution of genetic variation within and between populations. However, little attention has been paid to comparative analysis of groups of closely related species markedly different in their ecological requirements and evolutionary history. Such comparisons can provide critical information on the relative contributions played by gene flow, genetic drift, and perhaps selection, in determining genetic structure. Cave organisms are favorable organisms for this type of study for several reasons. Some species-groups provide graded series that include surface dwelling forms as well as cave-dwelling species. This allows comparisons between the genetic structure of epigean (noncave) and cave species. Moreover, the relative stability of the habitat, the small and relatively easily measured population sizes, and their discontinuous habitats make cave populations favorable material for studying genetic and spatial structuring (Sbordoni, 1980, 1982; Sbordoni et al., 1987).

In this study, 49 populations of North American cave crickets belonging to the genera *Hadenoecus* and *Euhadenoecus* have been analyzed electrophoretically at 41 gene loci. All nine species of these two genera have been studied. *Euhadenoecus* and *Hadenoecus* are the most conspicuous of the insects inhabiting caves in the Eastern United States (Fig. 1). These Raphidophorid cave crickets are the only members of the tribe Hadenoecini. The genus *Euhadenoecus* consists of four species: *E. adelphus, E. puteanus, E. fragilis,* and *E. insolitus.* Five species belong to the genus *Hadenoecus*: *H.*

barri, H. cumberlandicus, H. jonesi, H. opilionoides, and *H. subterraneus. E. adelphus* and *E. puteanus* are forest species; all the other members of the tribe Hadenoecini are obligate cavernicoles that reproduce only in caves, although they may emerge at night to feed (Hubbell and Norton, 1978 [and references therein]).

Comparative analysis of genetic structure of closely related species which have different kinds of ecological requirements, distribution patterns, and/or different degrees of isolation are clearest for six of the nine species: *E. puteanus, E. fragilis, E. insolitus, H. subterraneus, H. cumberlandicus,* and *H. opilionoides.* The first species is a forest species with a widespread distribution. The second occurs in areas where the limestone distribution is highly fragmented (Fig. 1; Davies, 1965). Its range is more restricted than that of *E. puteanus* but more widespread than those of other cave species. *E. insolitus* populations are characterized by a peculiar disjunct distribution (Fig. 1), with the northern populations geographically very close, although located in disjunct limestone formations (Varnedoe, 1973). The last three species, *H. cumberlandicus, H. opilionoides,* and *H. subterraneus* are obligate cave-dwellers with limited ranges (Fig. 1). Because of the different kinds of limestone configurations in the last three species' ranges, different degrees of isolation among populations are expected. *H. subterraneus* samples include populations located in fissured limestone areas, e.g., the Mammoth Cave region (Quinlan, 1970), together with some isolated populations in South-Central Kentucky. *H. cumberlandicus* occupies Eastern Kentucky caves in regions with different geological characteristics (Quinlan, 1970). *H. opilionoides* occupies a small area at the edge of the Cumberland Plateau. However, based on morphology two subspecies have been recognized, *H. o. opilionoides* in the North and *H. o. australis* in the South (Hubbell and Norton, 1978; Ives, 1930).

Finally, allele frequencies are used to assess intra- and interspecific genetic differences and infer the taxonomic relationships within the Hadenoecini tribe using genetic distances and multivariate analysis. These data are compared with the phylogeny based on morphology (Hubbell and Norton, 1978) and with data on DNA-DNA hybridization presented in an accompanying paper (Caccone and Powell, 1987).

Materials and Methods

Figure 1 shows distributions of the species and locations of the populations studied. Localities are identified in Table 1, together with a three-letter symbol by which they will be referred to subsequently. The majority are from sexual populations, although four are from parthenogenetic populations: BEC and HAS (*E. insolitus*) and BAT and DBO (*H. cumberlandicus*).

For each population at least 20 individuals were assayed for 41 gene loci, the only exception being NEW, for which only 12 individuals were studied. The enzymes studied are the following, with their abbreviations and number of loci scored in parentheses: adenylate kinase (AK, 1), aldolase (ALDO, 1), aldehyde oxidase (AO, 1), carbonic anhydrase (CA, 1), esterase (EST, 4), fumarase (FUM, 1), glutamate-oxaloacetic transaminase (GOT, 1), glucose-6-phosphate dehydrogenase (G6PD, 2), α-glycerophospate dehydrogenase (GPDH, 1), hydroxybutyrate (HBDH, 1), hexokinase (HK, 2), isocitrate dehydrogenase (IDH, 2), leucine amino peptidase (LAP, 1), malate dehydrogenase (MDH, 3), malic enzyme (ME, 1), mannose phosphate dehydrogenase (MPI, 1), peptidase (PEP, 6), 6-phosphoglucose dehydrogenase (6PGD, 1), phosphoglucomutase (PGM, 1), phosphohexose isomerase (PHI, 1), tetrazolium oxidase (TO, 1), triosephosphate isomerase (TPI, 1), xanthine dehydrogenase (XDH, 1), general proteins (PT, 5). Electrophoretic buffers, staining techniques, tissues used and the detailed allele frequencies are reported in Caccone (1986). Data were analyzed using the BIOSYS-1 program of Swofford and Selander (1981). Wright's F_{ST} (1951, 1965) was calculated to measure population differentiation. For loci with multiple alleles, a weighted average F_{ST} was calculated. Significance of the observed F_{ST} values was tested with a chi-square heterogeneity test (Snedecor and Irwin, 1933; Workman and Niswander, 1970). Gene flow estimates, Nm_W, were derived from F_{ST} values by the relationship $F_{ST} = 1/(1 + 4Nm)$, where m

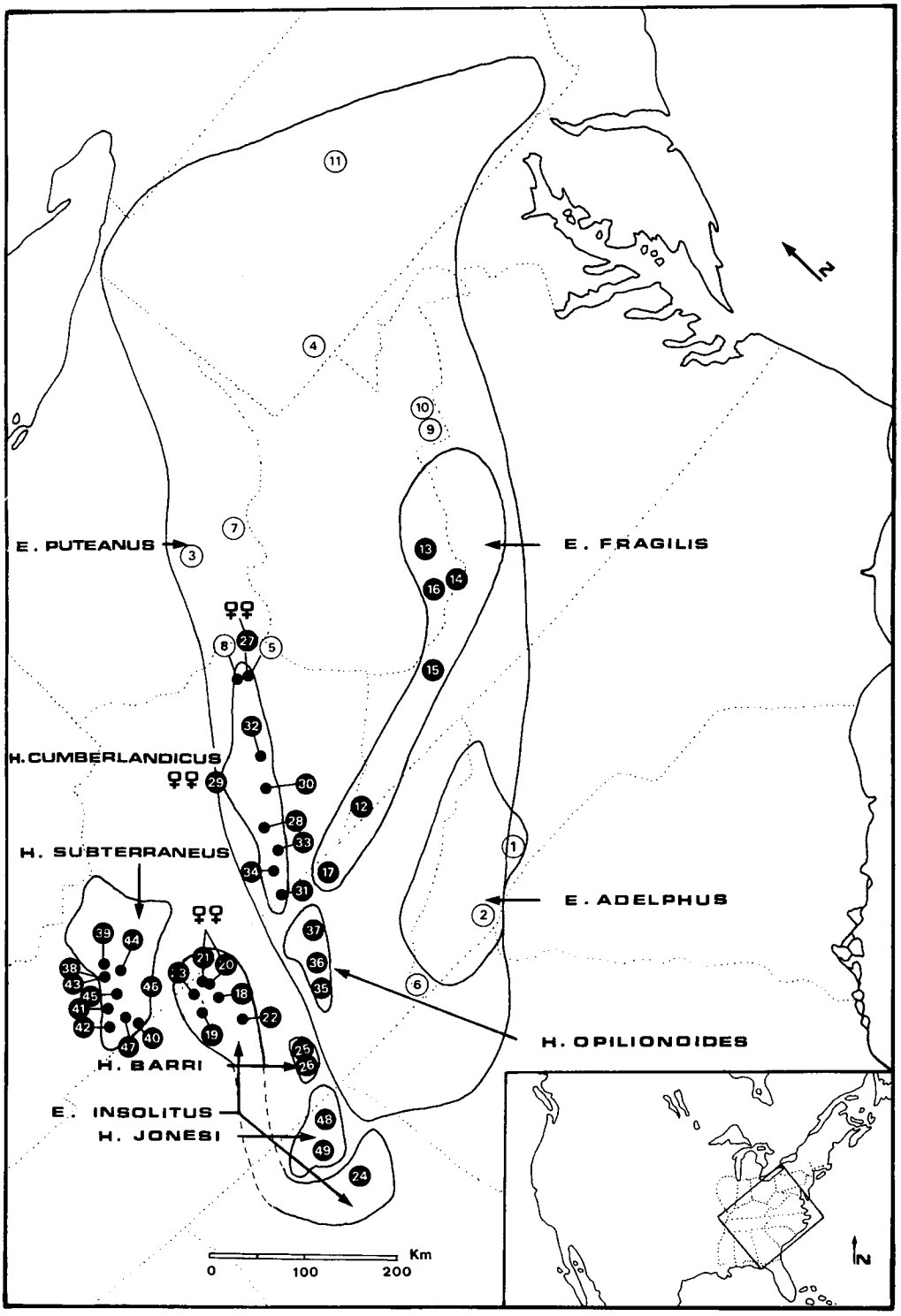

FIG. 1. Distributional pattern and collection sites for the nine species of Hadenoecini cave crickets. Numbers identify specific localities detailed in Table 1; white numbers refer to cave populations and black numbers refer to forest ones. ♀♀ refers to all-female parthenogenetic populations.

TABLE 1. List of *Euhadenoecus* and *Hadenoecus* populations studied. Numbers refer to localities illustrated in Figure 1.

E. adelphus
1) MON: Moonshiner's Cave, Henderson Co., NC
2) HIG: Highlands, Macon Co., NC

E. puteanus
3) ASH: Ash Cave State Park, Hocking Co., OH
4) BAR: Barton Cave, Fayette Co., PA
5) BAT: Bat Cave, Carter Cave State Park, Carter Co., KY
6) FON: Fontana Dam, Swain Co., NC
7) HOR: Horse Cave, Meigs Co., OH
8) LAU: Laurel Cave, Carter Cave State Park, Carter Co., KY
9) SEN: Seneca Caverns, Pendleton Co., WV
10) SMO: Smoke Hole Caverns, Grant Co., WV
11) WOD: Woodward Cave, Centre Co., PA

E. fragilis
12) GIL: Gilley Cave, Lee Co., VA
13) HBT: Higginbothams Cave, Greenbrier Co., WV
14) LIP: Lipps Cave, Greenbriar Co., WV
15) LOS: Lost Mill Cave, Tazewell Co., VA
16) MCL: McClungis Cave, Greenbrier Co., WV
17) NEW: New Mammoth Cave, Campbell Co., TN

E. insolitus
18) ANN: Ann White Cave, Macon Co., TN
19) BAC: Bat Cave, Sumner Co., TN
20) BEC: Aunt Beck Simmons Cave, Macon Co., TN
21) HAS: Hauskin's Cave, Macon Co., TN
22) IND: Indian Grave Point Cave, De Kalb Co., TN
23) MAS: Mason Cave, Sumner Co., TN
24) ARG: Argo Cave, Jefferson Co., AL

H. barri
25) CUM: Cumberland Caverns, Warren Co., TN
26) WON: Wonder Cave, Grundy Co., TN

H. cumberlandicus
27) BAT: Bat Cave, Carter Cave State Park, Carter Co., KY
28) BAK: Baker Cave, Pulasky Co., KY
29) DBO: Daniel Boone Cave, Jessamine Co., KY
30) HIS: Hisel Cave, Jackson Co., KY
31) KOG: Koger Cave, Wayne Co., KY
32) JRC: John Rogers Cave, Powell Co., KY
33) STA: Stab Cave, Pulasky Co., KY
34) WIN: Wind Cave, Pulasky Co., KY

H. opilionoides
35) BBC: Big Bone Cave, Van Buren Co., TN
36) BLF: Blind Fish Cave, White Co., TN
37) WOF: Wolf River Cave, Fentress Co., TN

H. subterraneus
38) FNM: Mammoth Cave, Frozen Niagara, Edmonson Co., KY
39) GON: Great Onyx Cave, Edmonson Co., KY
40) HOY: Hoy Cave, Simpson Co., KY
41) JAC: Jacks Cave, Warren Co., KY
42) JST: Jesse Stewart Cave, Butler Co., KY
43) MAM: Mammoth Cave, Marion Avenue, Edmonson Co., KY
44) PAR: Parkers Cave, Barren Co., KY
45) PRS: Pruytts Saltpeter Cave, Warren Co., KY
46) STH: Steep Hollow Cave, Warren Co., KY
47) WHE: Wheeler Cave, Logan Co., KY

H. jonesi
48) LIM: Limrock Blowing Cave, Jackson Co., AL
49) GRE: Doug Green Cave, Jackson Co., AL

is the migration rate and N the effective population size (Wright, 1931). Gene flow levels among populations were also estimated by Slatkin's methods (1981, 1985) based on the distribution of rare alleles (referred to as Nm^*, to distinguish them from Nm_W estimates). For each species, $\bar{p}(i)$ values were computed as average allele frequencies of the alleles present in the same number of populations (Slatkin, 1981). These values were plotted to produce qualitative gene-flow profiles. Quantitative estimates of the amount of gene flow were calculated following Slatkin's (1985) formula:

$$Nm^* = \frac{e^{\frac{-\ln[\bar{p}(1)] + 2.44}{0.505}}}{\frac{N}{25}}$$

where $\bar{p}(1)$ is the average frequency of all alleles found in only one population, and N is the average number of individuals sampled per population. This method was also used to obtain information on the population structure by computing $\bar{p}(1)$ and Nm^* values for different subsets of populations.

Genetic distances (D) were calculated using Nei's (1972), and Edwards' (1971, 1974) methods on the full data set. Dendrograms were produced from Nei's and Edwards' genetic distances with the UPGMA method of cluster analysis (Sokal and Michener, 1958), and the Wagner procedure (Farris, 1972; Swofford, 1981). Goodness of fit was evaluated by a cophenetic correlation coefficient (Sneath and Sokal, 1973). Correspondence analysis (AFC, Benzécri et al., 1973) was used to examine allele-frequency relationships among populations and species.

TABLE 2. F_{ST} indices in *Hadenoecus* and *Euhadenoecus*. See Caccone (1986) for abbreviation of loci and other details. *H. cu.* = *H. cumberlandicus*, *H. op.* = *H. opilionoides*, *H. su.* = *H. subterraneus*, *E. pu.* = *E. puteanus*, *E. fr.* = *E. fragilis*, *E. in.* = *E. insolitus*. Significance of population differentiation (indicated by asterisks) was tested with a chi-square heterogenity test.

Locus	*H. cu.*	*H. op.*	*H. su.*	Locus	*H. pu.*	*E. fr.*	*E. in.*
Ak	0.01	0.02	—	*Ak*	—	0.02	1.00**
Aldo	0.01	—	0.02	*Aldo*	0.02	0.01	—
Ao	0.03	0.07	0.04	*Ao*	0.03	0.04	0.18
Ca-2	0.03	0.70**	0.89**	*Ca-2*	0.04	0.09	0.02
Est-1	—	0.24	0.05	*Est-3*	0.09	0.66*	0.33
Est-3	—	0.01	0.68**	*Est-4*	0.19	0.98**	0.37
Est-4	0.69**	0.06	0.69**	*Est-5*	—	0.84*	0.10
Est-5	—	—	0.66**	*Fum*	0.06	0.06	0.03
Fum	0.05	0.02	0.85**	*G6pd-1*	—	1.00**	—
Got	0.03	0.04	—	*G6pd-2*	0.05	1.00**	0.96**
G6pd-1	0.01	—	0.93**	*Gpdh*	0.04	0.03	0.05
G6pd-2	—	0.10	1.00**	*Hbdh*	—	0.56*	0.18
Gpdh	—	0.49*	—	*Hk-1*	0.02	0.05	0.90**
Hbdh	0.68*	0.07	0.02	*Hk-2*	—	0.02	—
Hk-1	0.72**	0.04	0.08	*Idh-1*	—	0.06	0.93**
Hk-2	—	0.06	—	*Idh-2*	—	1.00**	—
Idh-1	0.87**	0.85**	0.03	*Lap*	0.36	0.17	0.25
Idh-2	0.03	—	0.01	*Mdh-0*	—	1.00**	—
Lap	0.07	0.13	0.09	*Mdh-1*	0.40	0.49*	0.08
Mdh-1	0.03	0.01	0.73**	*Mdh-3*	—	0.03	—
Mdh-3	—	0.01	0.02	*Me*	0.16	0.57*	0.04
Me	0.13	0.01	0.05	*Mpi*	0.77**	0.94**	1.00**
Mpi	0.32	0.02	0.02	*Pep-2*	0.18	—	—
Pep-2	0.01	0.03	0.01	*Pep-3*	—	—	0.01
Pep-3	0.47*	—	0.82**	*Pep-4*	0.09	0.08	0.08
Pep-4	0.50*	0.03	0.45*	*Pep-5*	0.14	0.03	0.03
Pep-5	0.53*	0.01	0.75**	*Pep-6*	—	1.00**	—
6Pgd	—	0.01	0.05	*6Pgd*	0.02	0.06	1.00**
Pgm	0.51*	0.02	0.08	*Pgm*	0.02	0.84**	0.02
Phi	0.07	0.05	0.03	*Phi*	0.05	0.98**	—
Xdh	0.06	0.15	0.97**	*Xdh*	0.20	0.04	0.23
Pt-1	0.01	—	—	*Pt-1*	—	1.00**	0.08
Pt-2	—	1.00**	—				
Pt-3	—	—	0.10				
Mean	0.46	0.30	0.58	Mean	0.24	0.72	0.44

* $P < 0.05$; ** $P < 0.01$.

RESULTS

F *Statistics Analysis and Gene-Flow Estimates Based on Rare Alleles*

Table 2 presents the results of the *F*-statistics analysis for *E. puteanus*, *E. fragilis*, *E. insolitus*, *H. cumberlandicus*, *H. opilionoides*, and *H. subterraneus*. F_{ST} measures the amount of differentiation among populations relative to the limiting amount under complete fixation. In the forest species *E. puteanus*, only the *Mpi* locus shows an F_{ST} value that is highly statistically significant. In contrast, cave species display several remarkably high mean F_{ST} values. The average F_{ST} value for cave species taken as a group is 0.50—twice that observed for *E. puteanus* (F_{ST} = 0.24). On average, half of the measured genetic variance is partitioned among populations of cave species. Estimates of gene flow (Nm_W) based on F_{ST} values are given in Table 3.

Figure 2 shows the results of a qualitative analysis of gene-flow levels in six of the Hadenoecini. The gene-flow profiles for the cave species are characterized by wide fluctuations and by high initial $\bar{p}(i)$ values. These curves very closely resemble those presented by Slatkin (1981) as typical of intermediate- and low-gene-flow species. The forest dwelling species *E. puteanus* is distinct from the other species because of the

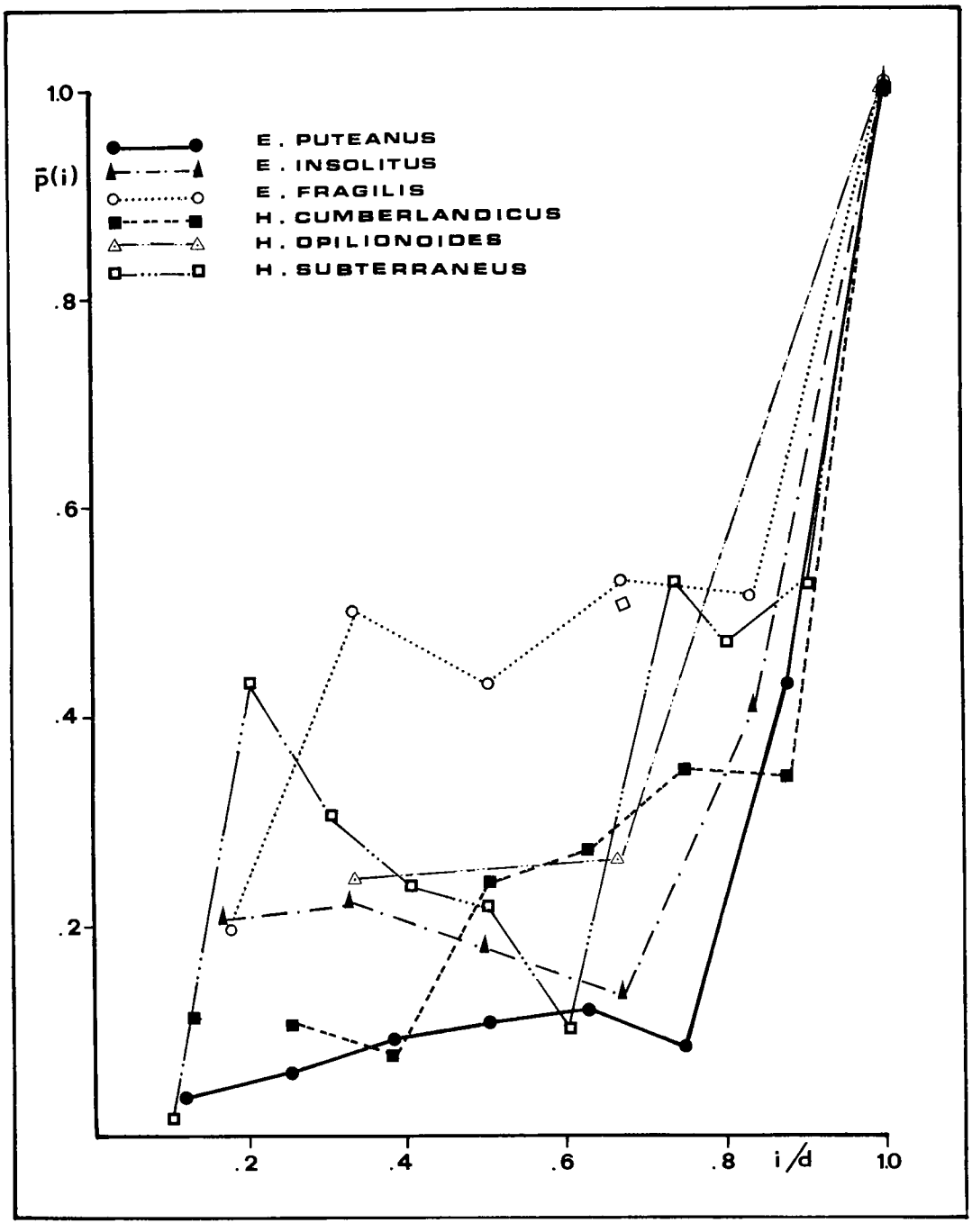

FIG. 2. Qualitative profiles of gene flow in six species of cave crickets. $\bar{p}(i)$ is the average conditional frequency of an allele, and i/d is its incidence, where i is the total number of populations it appears in and d is the total number of populations examined. The points plotted are average values of $\bar{p}(i)$ for all alleles having the same i/d values.

TABLE 3. Estimates of $\bar{p}(1)$ and Nm^* (Slatkin, 1981, 1985) in cave-crickets. $\bar{p}(1)$ is the average frequency of private alleles, Nm^* is the average number of migrants exchanged between local populations corrected for the sample size (Slatkin, 1985). Nm_W is the same estimate derived from F_{ST} values (Wright, 1931). G is the geographic distance in km. S, P, and n.p.a. refer to the mean number of individuals analyzed, the number of populations and the number of private alleles, respectively.

Species	S	P	n.p.a.	$\bar{p}(1)$	G	Nm^*	Nm_W
E. puteanus	31.8	9	13	0.15	304	0.27	0.79
E. fragilis	28.5	6	14	0.20	126	0.17	0.10
E. insolitus	25.1	7	20	0.29	117	0.09	0.32
H. cumberlandicus	32.2	8	16	0.11	200	0.49	0.29
H. opilionoides	28.1	3	14	0.24	50	0.12	0.58
H. subterraneus	30.1	10	8	0.02	83	13.89	0.18

lack of dramatic fluctuations and the very low $\bar{p}(i)$ values. This curve is similar to curves characteristic of high-gene-flow species (Slatkin, 1981). Table 3 lists, for the same six species, the average frequency of private alleles, $\bar{p}(i)$, the average geographic distance between populations, G, and quantitative estimates of gene flow based on Slatkin's (1985) method (Nm^*).

If Nm is smaller than one, genetic drift of neutral alleles may produce marked local differentiation; when Nm is greater than one, species tend to become effectively panmictic for neutral variants (Maruyama, 1970). Nm_W values are less than one both for the forest species *E. puteanus* and for the cave species. However, the forest species has the highest Nm_W value ($Nm_W = 0.79$), as predicted by its dispersal abilities. Slatkin's (1985) approach (Nm^*) yielded estimates that in two cases were in contrast with the predicted dispersal abilities of the species studied and with the Nm_W estimates. *E. puteanus,* the forest species, should have Nm values higher than any of the cave species. Yet, the estimated Nm^* value (0.27; Table 3) is similar, if not smaller, than the ones obtained for cave species. The second conflicting case is represented by *H. subterraneus.* Its estimated gene flow level ($Nm^* = 13.89$; Table 3) is surprisingly high for a cave species.

Population-Structure Analysis

Table 3 is based on the analysis of all data from a species. The result is a single estimate of Nm^* based on a single $\bar{p}(1)$ value. If isolated populations are included in the sample studied, the overall Nm^* estimate is inaccurate. However, since $\bar{p}(1)$ is relatively insensitive to the number of demes in the sample, more information about the genetic structure of the species can be gathered by obtaining $\bar{p}(1)$ estimates for different population subsets. If a population (or group of populations) is a genetic isolate, then the estimated Nm^* value of the remaining populations should be higher when the isolated population is removed. To explore this possibility Nm^* values were computed on different subsets of populations (Table 4).

If all nine populations are considered, gene flow levels in *E. puteanus* are low ($Nm^* = 0.27$; Table 4A). However, excluding the southernmost population (FON), the Nm^* is 4.67, an order of magnitude larger. Based on all the data together, we would conclude that there is a low level of gene flow in this species. However, the subsampling shows that there are probably high levels of gene flow among most populations. Table 4B illustrates the results for six populations of *E. fragilis.* The overall level of gene flow is low ($Nm^* = 0.17$) and remains low when either northern (LIP, MCL, and HBT) or southern (GIL, LOS, and NEW) populations are excluded, individually or in groups, indicating that all the populations studied are isolated. Similarly low Nm^* values are found among seven *E. insolitus* populations (Table 4C). Gene-flow levels among the seven populations studied are low ($Nm^* = 0.09$). The exclusion of the southern disjunct population, ARG, does not change the estimated gene-flow levels ($Nm^* = 0.17$). This species seems to be organized into isolates like *E. fragilis,* even though the average geographic distance between the northern populations (25 km) is much smaller than that between *E. fragilis* populations (126 km). Table 4D presents the results of the analysis for eight populations of *H. cumberlandicus.*

The overall level of gene flow is low ($Nm^* = 0.49$) and remains low when single populations or the northern groups of populations (BAT, DBO, JRC, and HIS) are excluded ($Nm^* = 0.36$). However, Nm^* estimates increase when the southern populations (BAK, KOG, STA, and WIN) are excluded ($Nm^* = 6.34$). Results from ten populations of *H. subterraneus* are summarized in Table 4E. Overall, gene flow seems unexpectatly high ($Nm^* = 13.89$), and it remains high when most of the populations are excluded. However, gene flow drops to values closer to estimates for the other cave species when five populations are removed (HOY, PAR, PRS, STH, and WHE). Table 4F reports results on the three *H. opilionoides* populations studied.

Genetic Distances

Table 5 lists intraspecific Nei's genetic distance (D) values together with average D values and their standard errors. Table 6 reports average coefficients of Nei's D between the nine morphologically inferred species of Hadenoecini. The estimates are means of pairwise comparisons between all populations of two species. The populations of Hadenoecini belonging to the same morphological species cluster together. This has not always been observed with cave crickets (e.g., the European sister group *Dolichopoda* [Allegrucci et al., 1986; Sbordoni et al., 1982, 1985, 1987]).

Genetic Relationships

Genetic-distance data, using both Nei's and Edwards' measures, were used to obtain UPGMA dendrograms. A phylogenetic network using Wagner's distance algorithm on Edwards' genetic distances was also analyzed. Since they all produced substantially the same topologies, only UPGMA dendrograms based on Nei's (1972) genetic distance are shown in Figure 3. The UPGMA and Wagner algorithms produced trees with similar goodness of fit, as measured by the cophenetic correlation (UPGMA: 0.991; Wagner Distance: 0.993). UPGMA clustering separates *E. puteanus, E. adelphus,* and *E. fragilis* from *E. insolitus* (Fig. 3A), and, within this group, the two forest-dwelling species, *E. puteanus* and *E. adelphus,* are more closely related than either is to *E. fragilis.* On the other hand, the Wagner tree places *E. insolitus* and *E. fragilis* equidistant from the two forest species. Intraspecific relationships seem to be determined by geographic proximity. The only exception involves *E. fragilis.* Using UPGMA, we find that the southernmost population (NEW) is clustered with one of the northern populations (HBT). Using the Wagner tree, we find that NEW is equidistant from the northern and the southern populations. Within *Hadenoecus, H. subterraneus* is the sister group of the *barri-cumberlandicus-jonesi-opilionoides* lineage. Within this grouping, *H. cumberlandicus* branches off first. Intraspecific clustering follows geography: pairs of closer populations are genetically more similar than are more distant ones, as in the other genus. The marginal and southern populations in *H. subterraneus* (HOY, STH, and WHE) branch off first from the central populations. Within the central populations, those occurring in isolated limestone outcrops (JAC, JST, and PRS) are genetically distinct from the others. The three populations of *H. opilionoides* are quite distinct genetic units, even though they are geographically close to each other.

The results of correspondence analysis for the four *Euhadenoecus* species are shown in Figure 4, and those for the five *Hadenoecus* species are shown in Figure 5. The first five axes are all statistically significant, together explaining 78.8% and 74.8% of the overall variance of the multidimensional system, for *Euhadenoecus* and *Hadenoecus,* respectively.

Discussion

Genetic Differentiation in the Hadenoecini Species

Using protein polymorphisms to infer levels of gene exchange does not distinguish between current and historical patterns. Especially in low-migration species, such as the cave species studied here, present-day distributions of protein variants may be more indicative of historical patterns of gene exchange than of current patterns (Larson et al., 1984; Caccone, 1985). The populations of the epigean species, *E. puteanus,* inhabiting territory that was covered with ice during the last Pleistocene glaciations (Howden, 1969) show little genetic differentiation, although the geographical area covered by these samples is large (average

TABLE 4. Numbers of private alleles (n.p.a.), sample sizes (numbers of individuals sampled), average frequencies of private alleles [$\bar{p}(1)$], and estimates of Nm^* obtained for different subsets of populations from six species of Hadenoecini cave crickets. Each population is in turn omitted, and the new Nm^* values are computed for the remaining populations. The estimates are based on 41 loci.

A) *Euhadenoecus puteanus* (nine populations, $Nm^* = 0.27$):

	Population excluded								
	ASH	BAR	BAT	FON	HOR	LAU	SEN	SMO	WOD
n.p.a.	11	12	13	11	14	14	12	11	18
Sample size	31.7	30.7	31.6	32.0	32.7	30.9	32.0	32.4	32.1
$\bar{p}(1)$	0.17	0.16	0.17	0.04	0.14	0.14	0.15	0.16	0.11
Nm^*	0.21	0.24	0.21	4.67	0.30	0.32	0.27	0.23	0.49

B) *Euhadenoecus fragilis* (six populations, $Nm^* = 0.17$):

	Population(s) excluded								
	GIL	HBT	LIP	LOS	MCL	NEW	LIP MCL	LIP MCL HBT	GIL LOS NEW
n.p.a.	14	13	20	12	22	15	23	13	16
Sample size	27.2	28.2	29.1	26.0	29.0	32.0	29.5	29.4	27.7
$\bar{p}(1)$	0.30	0.21	0.48	0.23	0.37	0.19	0.22	0.26	0.27
Nm^*	0.08	0.15	0.03	0.14	0.05	0.17	0.13	0.10	0.10

C) *Euhadenoecus insolitus* (seven populations, $Nm^* = 0.09$):

	Population(s) excluded								
	ANN	ARG	BAC	BEC	HAS	IND	MAS	ARG BAC	ARG IND
n.p.a.	19	13	14	19	21	21	21	8	11
Sample size	25.8	25.1	24.9	24.4	25.9	24.4	25.1	24.8	24.2
$\bar{p}(1)$	0.30	0.21	0.28	0.29	0.32	0.31	0.30	0.29	0.22
Nm^*	0.08	0.17	0.10	0.09	0.07	0.08	0.09	0.09	0.16

D) *Hadenoecus cumberlandicus* (eight populations, $Nm^* = 0.49$):

	Population(s) excluded									
	BAT	BAK	DBO	HIS	KOG	JRC	STA	WIN	BAT DBO JRC HIS	BAK KOG WIN STA
n.p.a.	14	15	15	15	13	14	13	13	14	9
Sample size	32.6	32.7	32.5	32.7	31.9	30.6	32.8	31.5	31.8	32.6
$\bar{p}(1)$	0.11	0.12	0.12	0.12	0.11	0.11	0.12	0.11	0.13	0.03
Nm^*	0.48	0.40	0.41	0.40	0.49	0.51	0.40	0.50	0.36	6.34

E) *Hadenoecus subterraneus* (ten populations, $Nm^* = 13.89$):

	Population excluded								
	FNM	GON	HOY	JAC	JST	MAM	PAR	PRS	STH
n.p.a.	6	9	10	10	9	8	9	9	10
Sample size	30.6	31.1	29.5	31.3	30.6	28.2	30.1	29.8	30.2
$\bar{p}(1)$	0.02	0.02	0.19	0.03	0.02	0.04	0.24	0.11	0.21
Nm^*	15.07	14.83	0.18	6.60	15.07	4.14	0.11	0.53	0.14

E) (continued):

	Population(s) excluded				
	WHE	HOY WHE	HOY STH WHE	HOY PRS STH WHE	HOY PAR PRS STH WHE
n.p.a.	10	9	10	9	10
Sample size	30.5	29.7	29.7	29.1	28.6
$\bar{p}(1)$	0.11	0.24	0.22	0.14	0.24
Nm^*	0.52	0.11	0.13	0.34	0.12

TABLE 4. Continued.

F) *Hadenoecus opilionoides* (three populations, $Nm^* = 0.12$):

	Population excluded		
	BBC	BLF	WOF
n.p.a.	17	16	17
Sample size	28.6	28.3	27.3
$\bar{p}(1)$	0.23	0.25	0.26
Nm^*	0.13	0.11	0.10

geographic distance among populations sampled is 253 km). On the contrary, cave species are more highly structured, even though the geographic distance between the populations studied is considerably lower than in *E. puteanus*. The smaller geographic units partition a greater portion of the total variation among populations than do the larger ones (Table 2). If gene flow among populations were a continuing phenomenon, it is unlikely that the geographically close populations would be more differentiated than are the distant ones (Tables 2–4).

The historical hypothesis—that forest populations recently radiated, while cave populations were isolated for longer periods of time—seems more likely than any explanation based on existing levels of gene flow. It is plausible that most cave populations or groups of cave populations were effectively isolated by events of the Pleistocene or earlier epochs (Hubbell and Norton, 1978) and that gene flow among them has not been restored. The existence of such isolates in *E. fragilis, H. cumberlandicus,* and *H. subterraneus* supports this argument.

Populations of *E. fragilis* are found in the Appalachian Ridge and Valley Province (Fig. 1). The cave systems are seldom extensive and are, on the whole, much more isolated than those of the interior plateaus where *H. subterraneus* and *H. cumberlandicus* occur (Hubbell and Norton, 1978). *E. fragilis* occupies many or most of the caves in a vast area of this region. Although epigean dispersal through forest habitats has been hypothesized (Hubbell and Norton, 1978), the high degree of interpopulation genetic differentiation ($F_{ST} = 0.72$, $Nm^* = 0.17$, $D = 0.206$) suggests that it must be very limited.

Populations of *H. cumberlandicus* live in caves of Eastern Kentucky, along the western ridge of the Cumberland Plateau and in adjacent parts of the Bluegrass Region. Gene flow between the two groups of populations ([BAT, DBO, HIS, JRC] and [BAK, KOG, STA, WIN]) is probably nonexistent, considering the high genetic distances between the two groups (Table 5D). The same reasoning is likely to hold for the populations of this species belonging to the southern group (BAK, KOG, STA, and WIN). These populations are geographically close (Fig. 1); yet, genetic distances between them are high (Table 5D), and gene-flow levels are low ($Nm^* = 0.36$; Table 4D). The fragmented limestone in this area may cause the high degree of differentiation. By contrast, the relatively high genetic uniformity found among the northern populations (BAT, DBO, HIS, and JRC; Table 5D; $Nm^* = 6.34$) is likely due to the more continuous structure of the limestone in this region (Quinlan, 1970).

The last species, *H. subterraneus,* is found in the Mammoth Cave region and other caves in South-Central Kentucky (Fig. 1). The high genetic uniformity among the populations from the Mammoth Cave area (GON, MAM, FNM, and PAR) reflects geological cohesiveness. Where the same species occurs in a fragmented limestone system (JAC, JST, STH, HOY, and WHE), it exhibits a high degree of genetic differentiation (Table 5F, Fig. 3B).

In summary, cave and forest species appear to differ in their levels of gene flow and, therefore, in their degrees of genetic differentiation. Among cave species, the degree of genetic structuring appears to be a consequence of limestone structure in the distribution range: *E. fragilis* populations are genetic isolates, inhabiting regions where the limestone is highly fragmented; *H. cumber-*

TABLE 5. Coefficients of genetic distance (Nei, 1972) between populations belonging to six species of Hadenoecini.

A) *E. puteanus* ($\bar{D}$ = 0.021 ± 0.003):

	ASH	BAR	BAT	FON	HOR	LAU	SEN	SMO
BAR	0.015	—						
BAT	0.025	0.015	—					
FON	0.070	0.043	0.046	—				
HOR	0.018	0.015	0.011	0.058	—			
LAU	0.028	0.017	0.002	0.042	0.013	—		
SEN	0.012	0.006	0.013	0.046	0.015	0.016	—	
SMO	0.013	0.008	0.009	0.047	0.011	0.011	0.004	—
WOD	0.017	0.012	0.003	0.048	0.006	0.005	0.011	0.007

B) *E. fragilis* ($\bar{D}$ = 0.206 ± 0.028):

	GIL	HBT	LIP	LOS	MCL
HBT	0.183	—			
LIP	0.317	0.163	—		
LOS	0.056	0.170	0.315	—	
MCL	0.368	0.199	0.029	0.361	—
NEW	0.105	0.120	0.258	0.157	0.296

C) *E. insolitus* ($\bar{D}$ = 0.080 ± 0.013):

	ANN	ARG	BAC	BEC	HAS	IND
ARG	0.166	—				
BAC	0.063	0.215	—			
BEC	0.012	0.174	0.083	—		
HAS	0.040	0.123	0.082	0.059	—	
IND	0.026	0.163	0.083	0.021	0.058	—
MAS	0.012	0.155	0.066	0.016	0.040	0.016

D) *H. cumberlandicus* ($\bar{D}$ = 0.078 ± 0.008):

	BAT	BAK	DBO	HIS	KOG	JRC	STA
BAK	0.076	—					
DBO	0.005	0.084	—				
HIS	0.039	0.024	0.045	—			
KOG	0.123	0.071	0.130	0.110	—		
JRC	0.026	0.074	0.032	0.047	0.116	—	
STA	0.118	0.029	0.126	0.061	0.093	0.111	—
WIN	0.144	0.042	0.149	0.081	0.053	0.130	0.054

E) *H. opilionoides* ($\bar{D}$ = 0.087 ± 0.014):

	BBC	BLF
BLF	0.109	—
WOF	0.091	0.062

F) *H. subterraneus* ($\bar{D}$ = 0.124 ± 0.012):

	FNM	GON	HOY	JAC	JST	MAM	PAR	PRS	STH
GON	0.024	—							
HOY	0.228	0.207	—						
JAC	0.073	0.090	0.300	—					
JST	0.051	0.071	0.207	0.129	—				
MAM	0.006	0.015	0.220	0.076	0.045	—			
PAR	0.007	0.021	0.224	0.077	0.034	0.003	—		
PRS	0.052	0.061	0.233	0.109	0.099	0.048	0.051	—	
STH	0.184	0.170	0.085	0.273	0.210	0.172	0.181	0.184	—
WHE	0.160	0.135	0.102	0.195	0.203	0.149	0.163	0.185	0.086

TABLE 6. Coefficients of genetic distance (Nei, 1972) between nine species of Hadenoecini. The estimates have been obtained as a mean of pairwise comparisons between all populations of two species. Leading-diagonal distance values refer to intraspecific genetic distance. Species abbreviations as in Table 2 with the additions of *E. ad.* = *E. adelphus*, *H. ba.* = *H. barri*, and *H. jo.* = *H. jonesi*.

	E. ad.	*E. pu.*	*E. fr.*	*E. in.*	*H. ba.*	*H. jo.*	*H. cu.*	*H. op.*	*H. su.*
E. ad.	0.096								
E. pu.	0.256	0.021							
E. fr.	0.604	0.511	0.206						
E. in.	0.563	0.634	0.535	0.080					
H. ba.	1.357	1.236	1.441	1.649	0.024				
H. jo.	1.314	1.270	1.609	1.739	0.375	0.024			
H. cu.	1.435	1.404	1.488	1.610	0.576	0.409	0.078		
H. op.	1.344	1.210	1.460	1.578	0.311	0.363	0.424	0.087	
H. su.	1.154	1.218	1.515	1.632	0.753	0.767	0.603	0.731	0.124

landicus and *H. subterraneus* inhabit areas of highly fissured limestone and have more genetically homogeneous populations; genetic isolates are found where isolated caves occur.

D, F_{ST}, *and* Nm*: *A Comparison*

It is difficult to compare findings based on different methodologies for determining population structure and levels of gene flow. Most commonly used genetic-distance (*D*) estimates are based on averages among monomorphic and polymorphic loci, F_{ST} values are based only on polymorphic loci, and Slatkin's *Nm** statistics are based on the distribution of private alleles, $\bar{p}(1)$. For North American cave crickets, *D* values (Table 5) and *Nm* measures based on F_{ST} (Nm_W, Table 3) are correlated with the predicted dispersal abilities (cave vs. forest species) and the degree of isolation between cave populations. On the contrary, Slatkin's *Nm** measures (Table 3) do not always reflect the predicted level of genetic differentiation. As Slatkin (1985) points out, the accuracy of his *Nm** estimates depends on several assumptions, including that populations are in genetic and demographic equilibrium and that the number of private alleles is large—Slatkin suggests a minimum of 20. Violation of these assumptions may account for the discrepancy between Nm_W and *Nm** for *E. puteanus* and *H. subterraneus*. Moreover, a single value of *Nm** for a species may be misleading if there is a great deal of spatial heterogeneity in levels of gene exchange among demes. A strength of Slatkin's method is its sensitivity in identifying cases in which the majority of populations sampled are exchanging (or have recently exchanged) genes at a high rate but one or a few populations are genetic isolates. This is clearly the case for *E. puteanus* and *H. subterraneus* (Table 5).

Only two other studies have compared the performance of *D*, F_{ST}, and *Nm** as estimators of levels of gene flow. Larson et al. (1984) studied gene-flow levels in several species of salamanders and found the three statistics to give approximately congruent results. Moreover, the authors stressed the relevance of these estimates in describing historical rather than current patterns of gene exchange in species with highly subdivided population structure, such as the salamander species they studied. Waples (1987) tested the performance of the same three statistics on several species of marine shore fishes. *Nm** based on Slatkin's method was not as strongly correlated with the dispersal abilities as were F_{ST} and *D* values. The poor performance of Slatkin's method in this case may be due to the low number of private alleles (<10 for each species) and to the small number of populations analyzed (<5). Moreover, eight out of the 10 species studied have *Nm** values above 10, which is outside the range of reliability of the method (Slatkin, 1985).

In summary, Slatkin's *Nm** method may provide reliable estimates of levels of past or current gene flow, if its limitations are taken into consideration. For high gene-flow species it is important to have a large number of private alleles; otherwise *Nm** estimates may be subject to a large stochastic

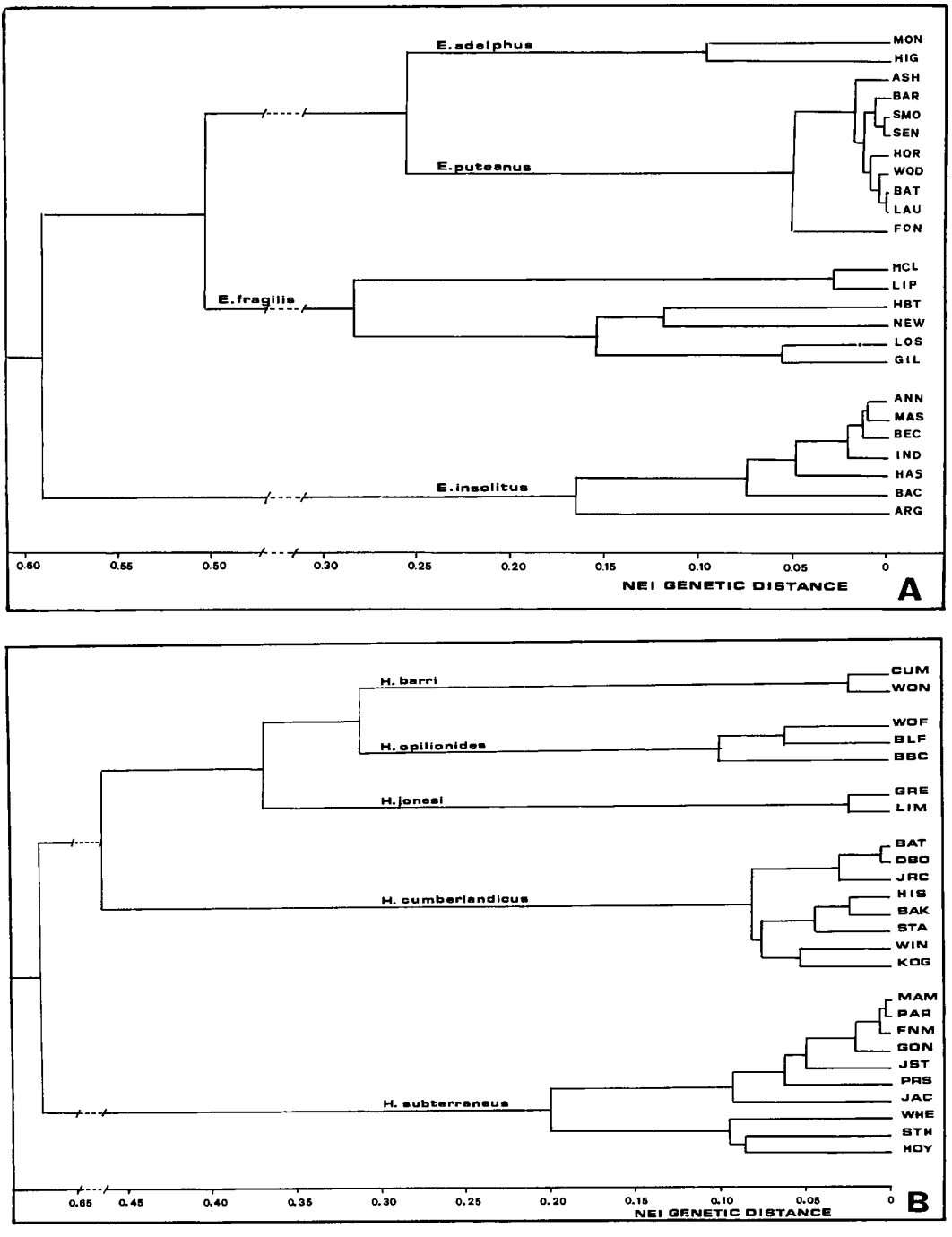

FIG. 3. UPGMA dendrogram based on Nei's genetic distance data in A) *Euhadenoecus* and B) *Hadenoecus* cave crickets.

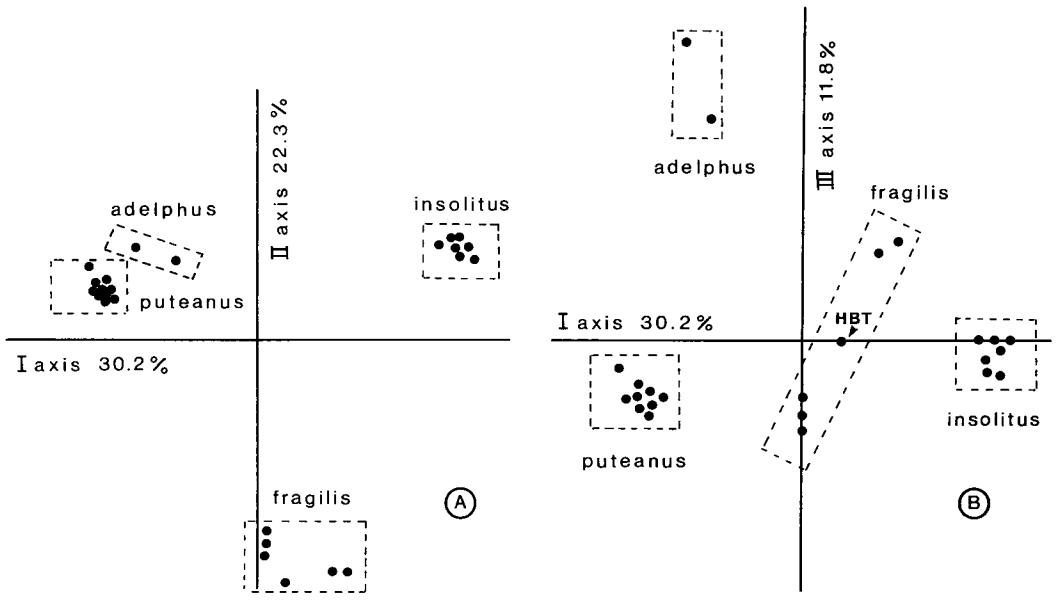

FIG. 4. Two-dimensional plot of 24 *Euhadenoecus* populations based on correspondence analysis at 41 gene loci. Points refer to the populations, and dashed lines enclose species. A) The reciprocal relationships of all the populations studied are indicated with respect to the multivariate space defined by the first and second axes; B) the reciprocal relationships of all the populations are indicated with respect to the multivariate space defined by the first and the third axes. Percentages refer to portion of the overall variance explained by each axis.

variance (Waples, 1987). For low gene-flow species, a detailed population-structure analysis is necessary to identify genetic isolates (Caccone, 1985).

Comparison to Other Cave Crickets

F_{ST} and Nei's D estimates are available for other populations of cave crickets. F_{ST} values for *Euhadenoecus* and *Hadenoecus* cave species (Table 2) are higher than those found in species of the closest relatives of the Hadenoecini, the European cave crickets, *Dolichopoda*. Average F_{ST} values in four species of this genus range from 0.15 to 0.25 (Sbordoni et al., 1985). In fact, the mean F_{ST} values reported in the present study are among the highest estimates found for outbreeding organisms (Larson et al., 1984 [and references therein]). However, broad comparisons of this type may be misleading, because of disparities among the loci tested and/or differences in geographic distributions and number of populations analyzed.

Intraspecific D values in the forest species, *E. puteanus,* are small (D = 0.021; Table 5A) and similar to those found in other noncave organisms (Ayala, 1975; Avise, 1976). These values are also comparable to those found for two other cave cricket species, which, like *E. puteanus,* are not strictly associated with caves (*Troglophilus cavicola* [Sbordoni et al., 1981] and *Ceuthophilus gracilipes* [Cockley et al., 1977]). Intraspecific D values in cave species are considerably higher than in *E. puteanus* (Table 5B–F). They are also higher than average intraspecific distance values in four *Dolichopoda* species (D = 0.099; Sbordoni et al., 1985). The relatively high genetic distances found between *E. fragilis* populations (Table 5B) and between the southern population (ARG) and the northern populations of *E. insolitus* (D = 0.166; Table 5C) may bring into question their taxonomic status. While it is difficult to make generalities about the precise degree of genetic differentiation associated with speciation events, there is clear evidence that Nei's D and degree of reproductive isolation are correlated in cave populations. The remarkably consistent evidence from *Dolichopoda* cave crickets, *Speonomus* Bathysciine beetles, and *Troglocharis* cave shrimps (Allegrucci et al., 1982; Cobolli Sbordoni et al.,

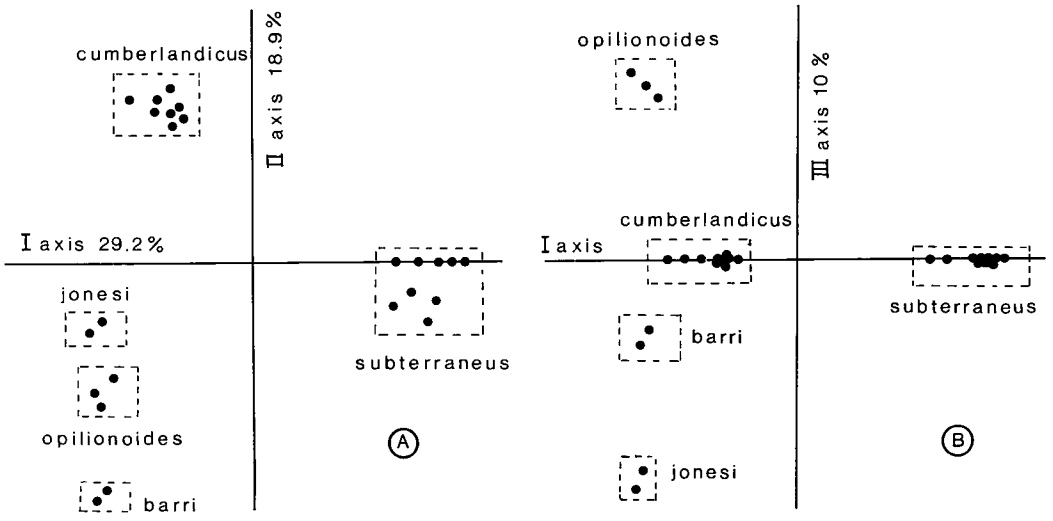

FIG. 5. Two dimensional plot of 25 *Hadenoecus* populations. Notations as in Figure 4.

1983; Delay et al., 1980; Sbordoni, 1982; Sbordoni et al., 1987) indicates that populations differing by *D*'s greater than 0.20 are reproductively isolated by postmating barriers. Thus, reproductive isolation may exist between some populations of *E. fragilis* and between some populations of *E. insolitus*. However, this hypothesis can be tested only by appropriate breeding experiments.

Genetic Relationships between Species and Phylogenetic Inferences

Within *Euhadenoecus,* cluster and multivariate analyses indicate that the forest species *E. puteanus* and *E. adelphus* are most closely related. The genetic affinities of *E. fragilis* and *E. insolitus* are less clear. UPGMA clusters *E. fragilis* with *E. puteanus* and *E. adelphus* (Fig. 3A). The Wagner tree and the correspondence analysis consider *E. puteanus-E. adelphus, E. fragilis,* and *E. insolitus* as three separate lineages equidistant from each other (Fig. 4A). Morphologically, *E. fragilis* and *E. insolitus* resemble each other but are so distant from the epigean lineage that their relative affinities are uncertain (Hubbell and Norton, 1978).

In the genus *Hadenoecus,* morphology places *H. cumberlandicus* with *H. subterraneus* (Hubbell and Norton, 1978), while isozyme data isolate *H. subterraneus* from all the other species (Figs. 3B, 5A, B). This latter arrangement is more consistent with geographical considerations. *H. cumberlandicus, H. barri, H. opilionoides,* and *H. jonesi* replace one another from north to south (Fig. 1), while *H. subterraneus* is located west of the other species. The relative positions of *H. barri, H. opilionoides,* and *H. jonesi* are also uncertain. Clustering of genetic-distance data places *H. barri* and *H. opilionoides* closer to each other than either of them is to *H. jonesi.* On the multidimensional space defined by the first three axes in the correspondence analysis *H. barri* and *H. jonesi* are the closest pair.

DNA-DNA hybridization data in the accompanying paper (Caccone and Powell, 1987) support the allozyme-based UPGMA tree, except in the case of the relative position of *H. jonesi.* DNA divergence data indicate that *H. jonesi* is the sister group of the *barri-cumberlandicus-opilionoides-subterraneus* lineage, while allozyme data place *H. subterraneus* as the sister taxon of the other four congeneric species. *H. jonesi* is the southernmost species of the genus (Fig. 1), and it may well have been the first one to separate from all the other taxa. Morphologically all five *Hadenoecus* species are so close that even their species status has been questioned (Hubbell and Norton, 1978). However, as explicitly acknowledged by Hubbell and Norton (1978), reconstructing phylogenies of cave organisms by mor-

phology alone is difficult, due to convergence and parallelism induced by selection in cave habitats. This problem would occur whether the method of analysis were phenetic, cladistic, or more "classical."

Regardless of whether the phylogenetic ambiguity is due to varying selection pressures on different traits (Turner et al., 1979) or related to the number of genes controlling their expression (Lewontin, 1984), the problem of choosing the most appropriate systematic tool for inferring phylogenies remains. Morphological analysis can produce misleading results if, for instance, the characters studied are under selective pressures. Phylogenetic inferences based on allozyme data generally overcome this problem but are limited by two factors. They reflect the evolutionary behavior of a very limited portion of the genome, and they are reliable estimators of genetic divergence only for closely related taxa. DNA-DNA hybridization studies of overall genetic divergence of single-copy DNA overcome these two biases (Gould, 1985; Sibley and Ahlquist, 1981). The accompanying paper (Caccone and Powell, 1987) presents intra- and inter-specific DNA divergence data for these cave crickets and compares the results with the isozyme and the morphological phylogenies.

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