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MOLECULAR EVOLUTIONARY DIVERGENCE AMONG NORTH AMERICAN CAVE CRICKETS. II. DNA-DNA HYBRIDIZATION

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Abstract.—Single-copy DNA divergence among 23 populations of cave crickets belonging to two genera (*Euhadenoecus* and *Hadenoecus*) has been determined by DNA-DNA hybridization employing the TEACL method. These same populations have been studied for allozyme variation (Caccone and Sbordoni, 1987). In addition, a European relative (*Dolichopoda laetitiae*) has been included as an outgroup for rooting the phylogeny. One of the most remarkable findings is the large degree of DNA divergence among these species and populations. A ΔT_m of up to 5°C has been found between populations of the same species; even further divergence is indicated by a lowered normalized percentage of reassociation. A phylogeny was constructed and tested for synchrony of rates, i.e., a molecular clock. Statistically, we could not reject the clock hypothesis. Attempts to calibrate the clock led to the conclusion that these insects are among the fastest evolving (with respect to single-copy DNA) groups yet studied—at least as fast as *Drosophila* and sea urchins—where a ΔT_m of 1°C indicates 0.5 to 1.5 MY since the last common ancestor. In general, the phylogeny derived from the DNA data agrees with that derived from isozymes. Nei's D and ΔT_m are correlated; in this group a D of 0.1 corresponds to a ΔT_m of about 1.5°C. This indicates that, relative to total single-copy DNA, the protein-coding regions of the genome are slowly evolving.

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Quantitative measurement of degrees of genetic differentiation between populations and species has long been a goal of evolutionary genetics. Several types of data, useful in different contexts, may be gathered. For example, population-genetic models require allele and genotype frequencies, as might come from electrophoretic surveys of protein variation or from determination of restriction-fragment length polymorphisms. For other purposes, such as systematics and studies of rates of molecular evolution, averages of differentiation over the total genome may be more useful. In the preceding paper (Caccone and Sbordoni, 1987) allozyme data for two genera of cave crickets were presented and analyzed in the context of population genetics. Here, we present data on the same populations and species for overall average differentiation as measured by DNA-DNA hybridization. The emphasis will be on constructing phylogenies, studying rates of DNA evolution in this group, and comparing DNA data with allozyme data.

Details of the natural history of the species were presented in the preceding paper (Caccone and Sbordoni, 1987) and will not be repeated here. Twenty-three of the 49 populations previously studied (Caccone and Sbordoni, 1987) were used in the DNA-DNA hybridization studies. They represent all nine species of the two genera of the North American Hadenoecini: *Euhadenoecus adelphus, E. puteanus, E. fragilis, E. insolitus, Hadenoecus jonesi, H. barri, H. opilionoides, H. cumberlandicus,* and *H. subterraneus.* In addition, for the DNA studies, we include as an outgroup *Dolichopoda laetitiae.* This species belongs to the European tribe Dolichopodini, which are considered to be the Hadenoecini's closest relatives (Hubbell and Norton, 1978).

The technique of DNA-DNA hybridization relies on the fact that double-stranded DNA becomes single-stranded when heated. The temperature at which the strands separate is determined by the fidelity of base pairing. Thus, homoduplexes (duplex DNA in which both strands were derived from

TABLE 1. List of all *Euhadenoecus, Hadenoecus,* and *Dolichopoda* populations studied and their three-letter abbreviations.

E. adelphus (forest-dwelling)
MON; Moonshiner's Cave, Henderson Co., NC
HIG; Highlands, Macon Co., NC

E. puteanus (forest-dwelling)
HOR; Horse Cave, Meigs Co., OH
SEN; Seneca Caverns, Pendleton Co., WV

E. fragilis (cave-dwelling)
GIL; Gilley Cave, Lee Co., VA
LIP; Lipps Cave, Greenbrier Co., WV
MCL; McClungis Cave, Greenbrier Co., WV
NEW; New Mammoth Cave, Campbell Co., TN

E. insolitus (cave-dwelling)
BEC; Aunt Beck Simmons Cave, Macon Co., TN
IND; Indian Grave Point Cave, De Kalb Co., TN
MAS; Mason Cave, Sumner Co., TN
ARG; Argo Cave, Jefferson Co., AL

H. barri (cave-dwelling)
CUM; Cumberland Caverns, Warren Co., TN

H. cumberlandicus (cave-dwelling)
BAT; Bat Cave, Carter Cave State Park, Carter Co., KY
HIS; Hisel Cave, Jackson Co., KY
KOG; Koger Cave, Wayne Co., KY
WIN; Wind Cave, Pulasky Co., KY

H. opilionoides (cave-dwelling)
BBC; Big Bone Cave, Van Buren Co., TN
BLF; Blind Fish Cave, White Co., TN

H. subterraneus (cave-dwelling)
MAM; Mammoth Cave, Marion Avenue, Edmonson Co., KY
WHE; Wheeler Cave, Logan Co., KY

H. jonesi (cave-dwelling)
LIM; Limrock Blowing Cave, Jackson Co., AL
GRE; Doug Green Cave, Jackson Co., AL

D. laetitiae (cave-dwelling)
DOL; Formello, Rome, ITALY

the same source) melt at a higher temperature than do heteroduplexes (duplex DNA in which the strands are from different sources), assuming there is some degree of genetic differentiation between the sources (i.e., different populations or species). The change in melting temperature can be directly related to base-pair mismatch (Britten et al., 1974). In addition to fidelity of base pairing, under most conditions, duplex stability is also determined by base composition: A-T bonds are weaker than G-C bonds. In the studies presented here, we have eliminated the base-composition effect by using the TEACL method of determining melting temperatures of DNA. While the TEACL technique has been used to study differentiation between sea urchins (Britten et al., 1978; Hall et al., 1980; Roberts et al., 1985) and *Drosophila* (Hunt et al., 1981; Hunt and Carson, 1983; Powell et al., 1986; Caccone et al., 1987), the present study is the most extensive application of this technique to date.

The power of DNA-DNA hybridization data to reconstruct phylogenies lies in the fact that one obtains an overall average divergence across the genome, i.e., over millions of base pairs. Two of the most important factors that can lead to inaccurate phylogenies, selection and chance, are virtually eliminated by the technique. At any given time, parts of a species' genome may be under selection to evolve rapidly, remain stable, or converge to sequences possessed by other species. However, such areas of the genome probably represent a very small part of the total, so that averaging across the total genome essentially eliminates misleading effects of selection. It is also nearly inconceivable that the sequence of millions of nucleotides would be similar due to chance, rather than common descent. As Gould (1985) pointed out, the complexity inherent in sequences of millions of nucleotides presents the best chance of distinguishing homology from analogy. Sibley and Ahlquist (1983) discuss in more detail the robustness of data from DNA-DNA hybridization studies for the construction of phylogenies.

A more controversial issue is whether data from DNA-DNA hybridization can be used as a molecular clock. We will address this issue and conclude that, with the statistical tests available, the molecular clock cannot be rejected. Further, in attempting to estimate a calibration for the clock, we conclude with Britten (1986*a*) that there is no single molecular clock that holds across all taxa. Cave crickets appear to be very rapidly evolving species (with respect to DNA), at least as fast as *Drosophila* and sea urchins.

MATERIALS AND METHODS

Table 1 lists the 24 populations of cave crickets utilized in this study, together with their geographic location and a three-letter

symbol by which they will be referred to subsequently. All nine species belonging to the tribe Hadenoecini are represented. The populations studied have previously been assayed electrophoretically for gene variation at the isozyme level, and the information on the species' distribution ranges and the populations' geographic locations are available in the preceding paper (Caccone and Sbordoni, 1987). When more than one population for each species have been utilized, the populations chosen have been the ones that, on the basis of morphology and isozyme analysis, were representative of the geographic variation of the species. *H. barri* is the only species in which only one population (CUM) has been analyzed, due to lack of material.

DNA Extraction and Sonication.—For each population, DNA was extracted from 10–30 frozen adult crickets belonging to both sexes. Crickets were ground in a sucrose-EDTA buffer (Goldring and Peacock, 1977), and the crude homogenate was passed through glass wool to remove debris. Nuclei were pelleted and resuspended in a 0.4 M EDTA, 10 mM Tris, and 2% sarkosyl, pH 9.5 lysis buffer. After 10 min of incubation at 65°C, proteins were digested with Proteinase K (100 μg/ml of lysate) for 3 hr at 50°C. After removing debris by centrifugation, CsCl (0.91 g/ml) and ethidium bromide (250 μg/ml) were added to the supernatant, and the solution was spun to equilibrium in an ultracentrifuge (36,000 rpm for 48 hr in a Beckman SW50 rotor). The fluorescent band was collected, and the ethidium bromide was extracted with butanol. The DNA was then ethanol precipitated and resuspended in H_2O, and its concentration was measured by spectrophotometry. Concentrations varied from 1 to 5 μg/μl. The DNA was then sonicated by using a high-intensity ultrasonic cell disruptor to obtain fragments ranging from 800 to 1,200 bp. These preparations represent the driver DNAs in each experiment.

DNA Labeling and Tracer Preparation.—One microgram was removed from the sonicated solutions and tritium-labeled by nick-translation (Maniatis et al., 1982). To obtain single-copy DNA, it is necessary to know the reassociation kinetics of the native DNA of the organisms under study. In this study, it was assumed that the percentage of repetitive DNA in cave crickets was similar to that found in other insects, namely 20–40% (Laird and McCarthy, 1969; Spradling and Rubin, 1981; Sperlin et al., 1976; Sohn et al., 1975). Single-copy tracer was prepared by dissociating the labeled DNA by heating. This was followed by reassociation in 0.48 M sodium phosphate buffer at 60°C to a C_0t of 50 (moles/liter)sec. Double-stranded DNA was removed by absorption to hydroxylapatite (Britten et al., 1974). Between 30% and 50% of the DNA bound to the column. The remaining fraction that did not bind was collected, dialyzed to remove the sodium phosphate, ethanol precipitated, pelleted by centrifugation, and resuspended in 100 μl distilled water. This fraction constitutes the tracer DNAs. Their specific activities varied between 1 and 15 × 10^6 cpm/μg.

Reassociation Conditions and Determination of Melting Temperatures.—Tracers were prepared from all the 24 populations, but, since specific activities varied considerably, not all possible pairwise combinations were studied. However, at least three melts were performed for each tracer (a homoduplex and two heteroduplex hybrid combinations). Methods for analyzing the thermal stability of duplex DNA molecules by S1 nuclease digestion in 2.4 M tetraethylammonium chloride (TEACL) are described in detail in Hall et al. (1980), Hunt et al. (1981), and Grula et al. (1982). Radioactively labeled tracers were reassociated with an excess of unlabeled driver DNA (1:1,000) to C_0t 6,000 or more in 1 M TEACL at 45°C. These conditions give a similar stringency as the one in phosphate buffer at 60°C (Orosz and Wetmur, 1977), namely, a base-pair match of about 75% or greater is required for stable duplex formation. Following reassociation, the duplex molecules were lightly digested with S1 nuclease at 37°C under conditions sufficient to remove 90% of the single-stranded material. An aliquot was saved for determining the length distribution of the S1-resistant tracer. The remaining solution was fractionated by Sephadex G-100 chromatography using 2.4 M TEACL as the elution buffer. Percentage reassociation was determined as the ratio of counts in the fractions

with intact duplexes to those in fractions with digested nucleotides.

The S1-resistant fraction was used for melting in a temperature block after adjusting the TEACL to 2.4 M with a refractometer. Thermal stability was assayed as described in Hunt et al. (1981). Briefly, the samples are divided in 100 μl fractions and placed in an aluminum block heated by circulating water at either end to give a linear temperature gradient. After 30 min, tubes are removed from the gradient and S1-digested (sufficient to give 99% digestion). The S1-resistent duplexes were precipitated, and the radioactivities in the supernatant and pellet were determined by scintillation counting. The percentage of radioactivity digested by nuclease S1 is then plotted against the melting temperatures to determine the melting curve. For all comparisons, a 12-point data set was obtained, and replicates were carried out for several temperature points (usually three or four). Between three and nine replicates were performed for the majority of the experiments.

Size Analysis of DNA Fragments.—Size distribution of the tracers after the first S1 digestion was determined following Hunt et al. (1981). The aliquots removed after the first S1 digestion were size-fractionated on a 3% alkaline agarose gel (McDonnell et al., 1977) using oligonucleotides obtained by digestion of pBR322 with *Hinf* I restriction endonuclease as markers. The T_m corrected for tracer length was calculated using the formula in Hall et al. (1980).

Statistical Analysis.—Percentage of reassociation and median melting temperatures are the two statistics commonly used to measure the degree of thermal stability of DNA duplexes. Percentage of reassociation was calculated as the percentage of the tracer that was not digestible by the first S1 nuclease treatment. Since a significant amount of the tracer is digestible by S1 in the heterologous reactions, the reaction percentages in these cases are normalized to that in the homoduplex, which is defined as 100. The median melting temperature (the temperature at which 50% of the single copy DNA that hybridized is dissociated) was determined by linear interpolation of the melting curves. This was done after having normalized the single-stranded DNA values with respect to two controls, one on ice and one heated to over 85°C, which represent 0% and 100% single-stranded (SS) DNA, respectively. Heteroduplex curves may start higher than homoduplex curves, because some melting occurs at the lowest temperatures of the block. Median melting temperatures (t_m) for the 12-points experiments and for replicates were averaged and the associated standard errors were calculated. These values were corrected by adding the temperature correction-factor obtained by the size analysis to produce corrected estimates of thermal stability, here symbolized as T_m's. Therefore, the symbol t_m refers to the uncorrected median melting temperature, while the symbol T_m refers to the same estimate corrected for the tracer's length. For a theoretical and empirical justification for this correction, see Hall et al. (1980).

The ΔT_m values (i.e., T_m of the homoduplex minus T_m of the heteroduplex from the same tracer DNA preparation) provide a quantitative measure of the overall level of divergence between the two taxa considered. These values were computed for all the experiments performed, along with their associated standard errors, by using the formula in Caccone et al. (1987).

Reciprocal comparisons (i.e., two hybridization experiments with each of the two taxa used in one reaction as tracer and in the other as driver) were carried out for most of the experiments to ensure that no major technical problems were present (theoretically they should yield the same results). Since reciprocity was holding for the vast majority of the experiments, some comparisons were carried out without reciprocals. The overall average ΔT_m values were computed for comparisons with reciprocal values, together with their respective standard errors, using the formula in Caccone et al. (1987).

A matrix of ΔT_m values was produced, using the averages of reciprocals whenever reciprocal comparisons were carried out or single values when reciprocals were not performed. This matrix was used for testing evolutionary rates, using a test available in the PHYLIP phylogenetic package (Felsenstein, 1985). This test compares the residual sums of squares between two trees of the same topology, both built by a least-squares

method (Cavalli-Sforza and Edwards, 1967). One tree-constructing algorithm assumes synchrony among lineages; the other does not. The comparisons are performed through a simple F test. If the residual sum of squares obtained from the synchronous tree (constancy assumed) is not significantly higher than the asynchronous tree (no constancy assumed), then the hypothesis of constancy of the rate of divergence cannot be rejected. Since this test cannot be performed on incomplete matrices, it was necessary to subdivide the principal matrix into 14 complete small matrices. The validity of generalizing from the smaller matrices to the principal matrix of ΔT_m values rests on the fact that all nodes and all branches were tested at least once. If the hypothesis of synchrony could not be rejected in any of these smaller matrices, then it is reasonably safe to draw the same conclusion for the overall matrix (J. Felsenstein, pers. comm.). Since the hypothesis of constancy in rates could not be rejected, the unweighted pair-group method using arithmetic averages (UPGMA; Sokal and Michener, 1958), was used on the overall matrix to summarize the phylogeny of the taxa.

Results

Melting Curves and Median Melting Temperatures

Examples of the melting profiles for hybrid DNA molecules between tracer and driver DNA from the same preparation (homoduplex) and for four heteroduplex comparisons with the same tracer are shown in Figure 1. The Appendix summarizes the results of all 606 experiments; in each table (A–X) all the experiments carried out with a particular tracer are reported. Replicates were carried out only for the melting curves; in most cases, only a single value is available for the fragment sizing and for the percentage of reassociation. Three to nine replicates for each homoduplex were carried out; their t_m's vary from 55.25°C to 58.24°C, with an average of 56.26°.

The standard errors associated with t_m values are around 0.15. The wide range in t_m values is mainly due to the different sizes of the tracers, since when these values are corrected by fragment size (T_m in our terminology), their average is 58.75°C with a standard error only of 0.12. Theoretically, perfectly matched duplexes in 2.4 M TEACL should have a T_m around 61°C (Britten et al., 1978). DNA-DNA hybridization studies of sea urchins using TEACL (Hall et al., 1980; Grula et al., 1982) present homoduplex T_m values around 60°C, but they were corrected for intrapreparation variability (heterozygosity) by adding 2°C (Britten et al., 1978). Considering that the present DNA preparations were made from populations of crickets, a similar correction is probably justified, which could bring the homoduplex T_m's as high as theoretically expected.

Fragment Size, T_m's, and ΔT_m's

Tracer size ranged around 200–300 base pairs. As expected, homoduplex tracers were generally larger than heteroduplex tracers. We have no explanation as to why heteroduplex tracers were occasionally longer than homoduplex tracers; this may have been due to variation in driver DNA lengths, which were not measured. Temperature corrections based on tracer length mostly ranged between 1.5° and 2.5°C. Heteroduplex comparisons in which tracers were longer than in the respective homoduplex and results with tracers shorter than 100 bp were included in the data set only if reciprocal comparisons with duplexes of reliable length were available and gave similar ΔT_m values. Replicates of the sizing experiment were carried out only in a few instances, because of the large number of cpm's needed for each experiment; the temperature correction in replicate experiments was repeatable to within 0.3°C.

A good example of the importance of the size correction is illustrated in the first entry in Table 4. The reciprocal Δt_m's for HIG and HOR are 3.82 and 7.97; when corrected for tracer length the reciprocal ΔT_m's are 4.88 and 4.43, with standard errors of 0.12 and 0.27, respectively.

Tables 2–6 list ΔT_m values, together with their standard errors. A good test of the reliability of ΔT_m for estimating levels of divergence is to check whether reciprocal experiments produce similar results. In Tables 2–6 Δt_m and ΔT_m values are given as weighted averages, computed as in Caccone et al. (1987), for cases in which reciprocal comparisons were carried out. Since t_m values

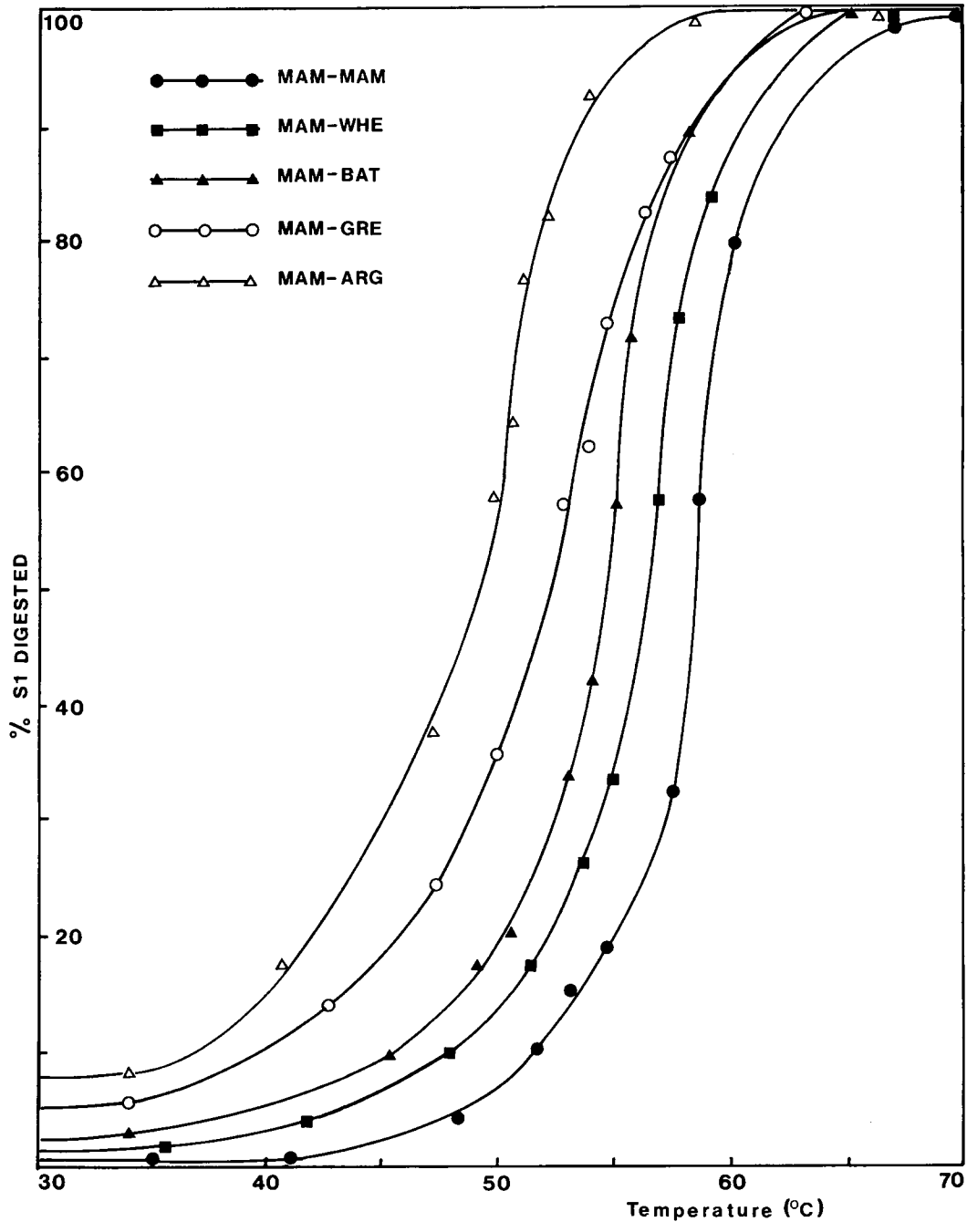

FIG. 1. Melting curves using MAM (*H. subterraneus*) as tracer. WHE is a conspecific population. BAT (*H. cumberlandicus*) and GRE (*H. jonesi*) are congeneric populations. ARG (*E. insolitus*) belongs to the other genus. Temperatures are on the x-axis; percentage of single-stranded DNA (% S1 Digested) is on the y-axis. Curves have been drawn "by eye" and are meant to serve only as visual aids.

TABLE 2. Intraspecific Δt_m and ΔT_m values in species of the genus *Euhadenoecus*. For measurements with reciprocal comparisons, each Δt_m and ΔT_m value is listed together with the weighted average (bar over population designations). *N* refers to the number of experiments and SE to the standard error.

Tracer-Driver	*N*	Δt_m (°C)	ΔT_m (°C)	SE
E. adelphus:				
HIG-MON	7	3.66	2.35	0.30
MON-HIG	3	2.55	2.16	0.17
$\overline{\text{HIG-MON}}$	10	2.81	2.20	0.15
E. puteanus:				
SEN-HOR	3	2.37	2.51	0.27
HOR-SEN	3	2.14	2.65	0.28
$\overline{\text{SEN-HOR}}$	6	2.26	2.58	0.19
E. fragilis:				
GIL-NEW	6	0.78	0.77	0.19
NEW-GIL	4	0.85	1.06	0.24
$\overline{\text{GIL-NEW}}$	10	0.81	0.88	0.15
GIL-MCL	6	3.37	3.66	0.22
MCL-GIL	3	4.54	4.89	0.25
$\overline{\text{GIL-MCL}}$	9	3.87	4.19	0.17
GIL-LIP	6	4.25	4.25	0.34
LIP-GIL	3	4.25	4.72	0.16
$\overline{\text{GIL-LIP}}$	9	4.25	4.64	0.15
LIP-MCL	9	1.56	2.10	0.23
MCL-LIP	3	2.73	2.91	0.20
$\overline{\text{LIP-MCL}}$	12	2.22	2.56	0.15
LIP-NEW	5	3.87	4.12	0.35
NEW-LIP	3	3.64	4.21	0.24
$\overline{\text{LIP-NEW}}$	8	3.71	4.18	0.20
MCL-NEW	3	4.41	4.71	0.16
NEW-MCL	5	3.84	3.62	0.29
$\overline{\text{MCL-NEW}}$	8	4.28	4.46	0.14
E. insolitus:				
MAS-IND	4	2.89	3.74	0.43
IND-MAS	6	2.30	3.07	0.34
$\overline{\text{MAS-IND}}$	10	2.53	3.33	0.27
MAS-BEC	3	0.81	2.08	0.29
BEC-MAS	6	0.87	2.94	0.32
$\overline{\text{MAS-BEC}}$	9	0.84	2.48	0.21
MAS-ARG	7	4.79	6.04	0.25
ARG-MAS	9	4.54	5.18	0.31
$\overline{\text{MAS-ARG}}$	16	4.69	5.71	0.19
IND-BEC	4	2.44	2.44	0.46
BEC-IND	3	2.33	4.30	0.21
$\overline{\text{IND-BEC}}$	7	2.35	3.99	0.19
IND-ARG	3	5.04	6.11	0.46
ARG-IND	6	4.61	5.13	0.23
$\overline{\text{IND-ARG}}$	9	4.70	5.33	0.21
BEC-ARG	3	4.31	6.49	0.38
ARG-BEC	6	4.65	5.48	0.32
$\overline{\text{BEC-ARG}}$	9	4.51	5.91	0.25

TABLE 3. Intraspecific Δt_m and ΔT_m values in species of the genus *Hadenoecus*. Symbols are as in Table 2.

Tracer-Driver	*N*	Δt_m (°C)	ΔT_m (°C)	SE
H. cumberlandicus:				
BAT-HIS	3	1.64	1.39	0.17
HIS-BAT	4	1.22	1.36	0.24
$\overline{\text{BAT-HIS}}$	7	1.49	1.37	0.14
BAT-WIN	3	4.57	2.45	0.28
WIN-BAT	3	2.22	2.15	0.24
$\overline{\text{BAT-WIN}}$	6	3.21	2.28	0.18
HIS-KOG	5	3.86	2.73	0.27
KOG-HIS	4	4.62	2.62	0.29
$\overline{\text{HIS-KOG}}$	9	4.21	2.68	0.19
HIS-WIN	3	3.10	2.40	0.24
WIN-HIS	3	1.54	1.92	0.20
$\overline{\text{HIS-WIN}}$	6	2.16	2.11	0.15
H. opilionoides:				
BBC-BLF	3	3.26	2.24	0.22
H. jonesi:				
GRE-LIM	3	0.53	0.51	0.15
LIM-GRE	3	—	0.52	0.21
$\overline{\text{GRE-LIM}}$	6	0.53	0.51	0.12
H. subterraneus:				
MAM-WHE	3	2.35	2.10	0.26
WHE-MAM	3	1.42	2.07	0.25
$\overline{\text{MAM-WHE}}$	6	1.88	2.08	0.18

are dependent on tracer length, T_m and ΔT_m values are more accurate and will be considered in more detail. ΔT_m values are 2.20°C and 2.58°C between populations of *E. adelphus* and *E. puteanus*, respectively. Among the four populations of *E. fragilis*, ΔT_m's varied from as little as 0.88°C to 4.64°C. Comparable variation occurs among *E. insolitus* populations, where values range from 2.48°C to 5.91°C (Table 2). Considering *Hadenoecus*, among the four populations of *H. cumberlandicus* ΔT_m's range from 1.37°C to 2.68°C. The two populations of *H. jonesi* have the smallest ΔT_m (0.51°C), while a much higher ΔT_m is found between the two *H. opilionoides* (ΔT_m = 2.24°C) and *H. subterraneus* (ΔT_m = 2.08°C) populations (Table 3).

Interspecific comparisons within the genus *Euhadenoecus* (Table 4) produced ΔT_m's ranging from 4.44°C to 4.92°C between *E. adelphus* populations (HIG and MON) and *E. puteanus* populations (HOR and SEN). ΔT_m's of any of these four populations with any of the four populations of *E. fragilis*

TABLE 4. Interspecific Δt_m and ΔT_m values for the genus *Euhadenoecus*. Symbols are as in Table 2.

Tracer-Driver	N	Δt_m (°C)	ΔT_m (°C)	SE
HIG-HOR	6	3.82	4.88	0.12
HOR-HIG	4	7.97	4.43	0.27
HOR-HIG	10	4.48	4.81	0.11
HIG-SEN	6	3.47	4.92	0.10
SEN-MON	6	4.70	4.44	0.23
HIG-MCL	4	6.01	6.84	0.20
MCL-HIG	5	9.89	6.83	0.16
HIG-MCL	9	7.48	6.83	0.16
HIG-NEW	3	5.82	6.66	0.19
NEW-HIG	7	8.88	6.14	0.25
HIG-NEW	10	6.91	6.47	0.15
SEN-NEW	4	8.18	6.92	0.18
HIG-ARG	3	8.37	8.85	0.12
ARG-HIG	4	12.78	8.55	0.20
HIG-ARG	7	9.46	8.77	0.10
HIG-IND	4	7.21	8.70	0.17
IND-HIG	3	11.70	8.43	0.26
HIG-IND	7	8.62	8.61	0.14
HIG-MAS	4	7.55	8.62	0.27
MAS-HIG	4	11.14	8.66	0.26
HIG-MAS	8	9.41	8.64	0.19
MON-ARG	2	8.72	8.28	—
ARG-MON	3	9.60	8.29	0.25
MON-ARG	5	9.16	8.28	—
SEN-IND	3	14.80	8.35	0.20
SEN-MAS	4	15.38	8.34	0.20
MAS-SEN	5	8.37	8.12	0.26
SEN-MAS	9	12.66	8.25	0.16
HOR-ARG	5	9.42	7.91	0.30
ARG-HOR	4	9.46	8.56	0.24
HOR-ARG	9	9.44	8.30	0.19
HOR-IND	4	11.27	8.87	0.24
GIL-SEN	5	6.83	6.53	0.18
GIL-ARG	4	8.84	8.36	0.23
MCL-HOR	4	8.10	7.50	0.31
MCL-SEN	4	7.74	7.51	0.19
MCL-IND	4	8.23	7.83	0.17
MCL-MAS	4	8.36	8.34	0.27
MAS-MCL	2	8.23	7.87	0.31
MCL-MAS	7	8.30	8.14	0.20
NEW-HOR	4	6.92	6.61	0.34
NEW-IND	4	9.83	8.19	0.23
NEW-MAS	5	7.63	7.63	0.24
MAS-NEW	4	8.33	8.45	0.28
NEW-MAS	9	7.92	7.98	0.18
MAS-HOR	5	8.55	8.22	0.32
MAS-MON	4	9.25	8.65	0.25
ARG-MCL	4	8.22	8.27	0.22

TABLE 5. Interspecific Δt_m and ΔT_m values for the genus *Hadenoecus*. Symbols are as in Table 2.

Tracer-Driver	N	Δt_m (°C)	ΔT_m (°C)	SE
CUM-BBC	3	2.95	2.72	0.24
CUM-BAT	3	4.22	4.08	0.32
CUM-HIS	2	5.00	4.64	—
CUM-MAM	3	5.97	5.52	0.31
CUM-GRE	6	7.42	7.44	0.40
BAT-BBC	3	4.39	4.27	0.21
BBC-BAT	4	4.55	4.34	0.27
BAT-BBC	7	4.45	4.30	0.17
BAT-MAM	3	6.62	6.38	0.40
MAM-BAT	4	5.84	5.66	0.19
BAT-MAM	7	5.98	5.79	0.17
HIS-BBC	4	4.13	3.93	0.19
BBC-HIS	3	4.77	3.69	0.29
HIS-BBC	7	4.32	3.86	0.16
HIS-WHE	3	5.30	5.01	0.29
WHE-HIS	3	5.59	5.29	0.22
HIS-WHE	6	5.48	5.19	0.17
HIS-GRE	3	7.55	7.75	0.17
GRE-HIS	3	7.27	7.17	0.11
HIS-GRE	6	7.35	7.33	0.09
HIS-LIM	3	8.51	8.29	0.14
LIM-HIS	3	8.19	6.80	0.32
HIS-LIM	6	8.46	8.06	0.13
KOG-MAM	4	6.12	5.98	0.30
BBC-MAM	3	6.54	5.28	0.32
MAM-BBC	4	5.88	5.58	0.17
BBC-MAM	7	6.03	5.51	0.15
BBC-WHE	4	5.98	5.18	0.26
WHE-BBC	4	5.40	5.92	0.25
BBC-WHE	8	5.68	6.57	0.18
BBC-GRE	3	8.51	7.50	0.36
GRE-BBC	5	7.53	7.00	0.20
BBC-GRE	8	7.76	7.12	0.17
BBC-LIM	3	8.48	8.08	0.29
LIM-BBC	3	5.10	7.16	0.21
BBC-LIM	6	6.24	7.47	0.17
BLF-MAM	2	5.92	4.73	—
GRE-MAM	4	7.89	7.05	0.14
MAM-GRE	4	7.52	7.78	0.26
GRE-MAM	8	7.81	7.21	0.12
LIM-MAM	3	7.81	8.38	0.39

(GIL, LIP, MCL, and NEW) range from 6.47°C to 7.51°C. Comparisons among all eight of the preceding populations and the four populations of *E. insolitus* (BEC, IND, MAS, and ARG), yield ΔT_m's ranging from 7.83°C to 8.87°C (Table 4). Within *Hadenoecus*, the lowest interspecific ΔT_m (2.72°C) is found between one of the *H. opi-*

TABLE 6. Intergeneric Δt_m and ΔT_m values between *Hadenoecus, Euhadenoecus,* and *Dolichopoda.* Symbols are as in Table 2.

Tracer-Driver	*N*	Δt_m	ΔT_m	SE
HIS-HIG	3	11.18	9.87	0.25
HIS-MON	3	11.80	11.19	0.15
HIS-GIL	3	9.73	9.88	0.19
GIL-HIS	3	10.18	10.02	0.12
HIS-GIL	6	10.04	9.98	0.10
HIS-MCL	3	11.05	10.64	0.20
HIS-ARG	3	12.16	11.41	0.29
WIN-MON	2	11.05	10.29	—
BBC-ARG	3	11.48	9.78	0.28
ARG-BBC	2	10.97	10.45	—
BBC-ARG	5	11.22	10.11	—
GRE-MON	6	10.20	9.53	0.32
GRE-GIL	4	10.50	10.07	0.40
GIL-GRE	3	10.05	9.91	0.21
GRE-GIL	7	10.15	9.94	0.18
GRE-ARG	4	12.98	10.58	0.26
MAM-ARG	3	13.77	10.76	0.20
HIG-BAT	3	9.47	10.96	0.10
MCL-BAT	4	9.72	9.70	0.18
ARG-BAT	8	8.63	9.29	0.38
DOL-BAT	3	19.98	18.76	0.55
BAT-DOL	1	19.05	17.38	—
DOL-BAT	4	19.51	18.07	—
DOL-MON	3	17.74	17.58	0.32
DOL-WIN	2	18.71	17.28	—

lionoides populations (BBC) and the *H. barri* population (CUM). When populations of either species (BBC, BLF, and CUM) are hybridized with any of the four *H. cumberlandicus* populations (BAT, HIS, KOG, and WIN), ΔT_m values range from 3.86°C to 4.64°C. ΔT_m's between any of the preceding populations and either of the two populations of *H. subterraneus* (MAM and WHE) range from 4.73°C to 5.98°C. When the two *H. jonesi* populations (GRE and LIM) are hybridized with any congeneric population, the T_m's range from 7.12°C to 8.38°C (Table 5).

ΔT_m's between populations belonging to different genera of the Hadenoecini (only *H. barri* is missing) range from 9.29°C to 11.41°C. ΔT_m's between populations belonging to either Hadenoecini genus and the outgroup, *D. laetitiae,* range from 17.28°C to 18.07°C (Table 6).

Standard errors of all these estimates are low. They vary from 0.09 to 0.34. Table 7 lists the average of the standard errors in different ranges of ΔT_m values. Standard errors vary from 0.13 to 0.23 over a wide range of ΔT_m's (0.1–11.9). The only exception (ΔT_m range: 17.0–19.0; average SE = 0.43) involves comparisons of Hadenoecini with the European outgroup, *D. laetitiae.* This result is not unexpected, since such high values of ΔT_m are close to the reliability limits of this measure. These low standard errors make it possible to discriminate among populations within all the species studied and to assess the interspecific and intergeneric relationships with no ambiguities. However, it should be pointed out that these standard errors are minimum estimates, since they refer only to the error associated with the temperature gradients. They do not take into consideration other sources of error, such as the first S1-nuclease digestion or the fragment sizing.

TABLE 7. Averages of standard errors (SE) for different ranges of ΔT_m values. *N* refers to the number of ΔT_m values in that range.

Range of ΔT_m	*N*	Mean SE
0–0.9	2	0.13
1.0–1.9	1	0.14
2.0–2.9	10	0.19
3.0–3.9	3	0.21
4.0–4.9	9	0.18
5.0–5.9	9	0.21
6.0–6.9	5	0.20
7.0–7.9	9	0.20
8.0–8.9	16	0.22
9.0–9.9	6	0.23
10.0–10.9	3	0.19
11.0–11.9	2	0.22
17.0–19.0	2	0.43

Tree Clustering and Evolutionary Clock Test

Felsenstein's (1984) test was run on 14 complete matrices extracted from the main data set of ΔT_m's to study evolutionary rates in these cave crickets. These represent all the complete matrices in the data set, i.e., no selection of matrices was performed. All branches and nodes were included. The matrices tested were: 1) the full 4 × 4 ΔT_m matrix of the *E. insolitus* populations, to test rates within this species; 2) the full 4 × 4 matrix of the *E. fragilis* populations, to test rates within this species; 3) a 4 × 4

matrix with HIG, MON, SEN, and MAS, to test rates among *E. adelphus, E. puteanus,* and *E. insolitus*; 4) a 4 × 4 matrix with HOR, SEN, HIG, and MAS, to test the same node with different populations; 5) a 4 × 4 matrix with HIG, HOR, NEW, and IND, to check rates among *E. adelphus, E. puteanus, E. fragilis,* and *E. insolitus*; 6) a 3 × 3 matrix with HIG, MCL, and ARG, to test the same node as matrix 5 but with different populations; 7) a 4 × 4 matrix with CUM, BAT, HIS, and BBC, to test rates among *H. barri, H. cumberlandicus,* and *H. opilionoides*; 8) a 4 × 4 matrix with GRE, LIM, MAM, and BBC, to test rates among *H. jonesi, H. subterraneus,* and *H. opilionoides*; 9) a 3 × 3 matrix with BBC, BLF, and MAM, to test the rate between *H. opilionoides* and *H. subterraneus*; 10) a 4 × 4 matrix with CUM, BBC, BAT, and MAM, to test rates among *H. barri, H. opilionoides, H. cumberlandicus,* and *H. subterraneus*; 11) a 5 × 5 matrix with BAT, HIS, MCL, ARG, and HIG, to test rates between the two genera, using *H. cumberlandicus, E. fragilis, E. insolitus,* and *E. adelphus*; 12) a 3 × 3 matrix with ARG, HIS, and GRE, to check the same node as matrix 11, but with *E. insolitus, H. cumberlandicus,* and *H. jonesi*; and 13 and 14) two 3 × 3 matrices with DOL, BAT, and WIN, and DOL, MON, and WIN, respectively, to check rates between both North American genera and the outgroup.

For each of these matrices, several tree topologies were obtained by successive runs of two programs in the PHYLIP package: "Kitsch," which assumes equality in sister branch lengths (i.e., molecular clock), and "Fitch," which does not. The distance algorithm used was that of Cavalli-Sforza and Edwards (1967), since standard errors do not increase with greater ΔT_m values (Table 7) (Felsenstein, 1984). The residual sums of squares between trees having the same topology were compared with an F test. In all cases the null hypothesis holds; the increase in the residual sum of squares (resulting from the reduced number of parameters that the synchronous-tree algorithm utilized) is not significant. Thus, the molecular clock hypothesis (synchrony of rates of change in all branches) cannot be rejected. While one may argue that using reduced matrices is not a powerful test of overall constancy, the fact that none of the 14 matrices tested was significant (by the one out of 20 criterion) adds confidence in accepting the assumption. Furthermore, the methodology for testing incomplete matrices that has recently been developed (Sheldon, 1987) was applied to these data with the same result, i.e., synchrony could not be rejected (Powell and Caccone, unpubl.). On the basis of these results UPGMA dendrogram has been constructed assuming average values for the missing entries (Fig. 2).

Percentage Reassociation

Percentage reassociation, like the ΔT_m, should reflect degree of genetic divergence (Sibley and Ahlquist, 1981; Britten, 1986*a*). The percentage reassociation of homoduplexes ranges from 70.4% to 98.6%. The normalized percentage reassociation (NPR) for intraspecific heteroduplexes ranged from 63.5% to 100%; for interspecific heteroduplexes the range was 27–96.6%; for intergeneric heteroduplexes the range was 40.0–64.5%. The NPR between the North American and European species is 41.8%.

Unfortunately, repeatability of this statistic is not as good as for T_m. We performed replicate reassociation reactions for 12 homoduplex and 12 heteroduplex comparisons. For five of these comparisons, three to five replicates were performed; standard errors of percentage reassociation ranged from 0.5% to 8.6%. For 19 of the comparisons, only two determinations were made; the differences ranged from 0.04% to 20.5% with a mean of 7.8%. Thus the error associated with this statistic for any single measurement is on the order of 10%.

Despite these problems, the pattern of NPR is far from random: the more distantly related the two taxa are, the lower the NPR. Figure 3 is a graph of NPR against ΔT_m. Clearly, as the ΔT_m increases, NPR decreases. The slope of the linear-regression line is -4.48 for cave crickets, not including the intertribal point. This near linearity of relationship has been observed in other groups (e.g., Schulze and Lee, 1986).

Another aspect of genome evolution that may be reflected in NPR is change in genome size. When reciprocal reactions give asymmetrical NPR's, one can infer a difference in the size of the single-copy fraction

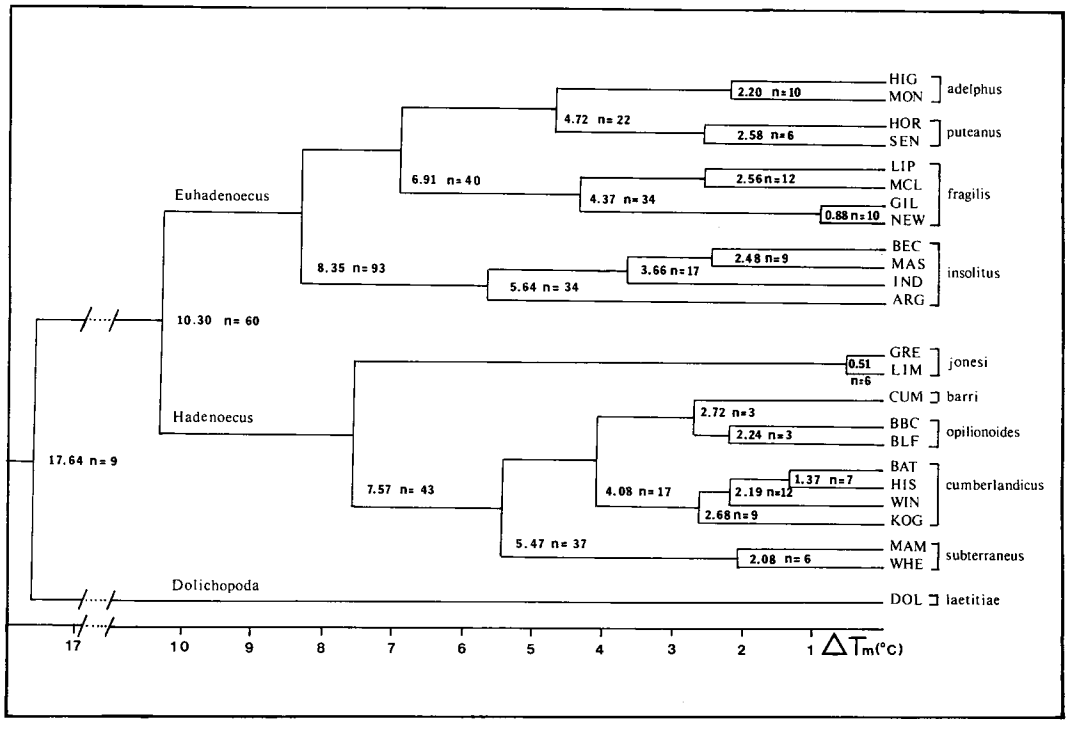

FIG. 2. UPGMA tree based on the ΔT_m values among the 24 populations of cave crickets. For each node, the average ΔT_m are reported together with the number of comparisons (n) carried out for that node.

(R. Britten, pers. comm.). For example the BAT-BBC NPR is 88.5% and that for BBC-BAT is 60.2%, giving a ratio of 0.68, which suggests the single-copy DNA content of BAT is 32% less than BBC. One can do this for all of the reciprocal cases and rank the DNA content. The resulting data are reasonably consistent. However, because we are not certain of the reliability of these estimates, we have not presented the graphs here. Nevertheless, there is clear indication in these data of quite large variation in single-copy genome size among these very closely related insects.

Single-Copy Divergence Versus Isozyme Divergence

Figure 4 compares ΔT_m's (x-axis) with estimates of divergence (Nei's D; y-axis) based on the isozyme study (Caccone and Sbordoni, 1987) for the same pairwise comparisons. For intra- and interspecific comparisons, Nei's D values of as little as 0.05 can correspond to a ΔT_m as high as 2. More generally, however, it seems that a D of 0.1 corresponds to a ΔT_m of about 1.5°. For the intergeneric comparisons, this correspondence does not hold; Nei's D's from 1 to 1.7 correspond to a smaller range of ΔT_m's (10–12°C). This result is not unexpected, since it is known that values of Nei's D above 1 suffer a saturation effect and therefore are unreliable, even as indicators of isozyme divergence. Alternatively, and not mutually exclusively, ΔT_m may be slowing as a metric due to low NPR and approach to the limit of reliability of measurement.

Nei's genetic distance can be roughly translated to percentage of base-pair differencers. If an average gene length of 1,000 bp is assumed and that one in five bp changes results in a change in the net electric charge of a protein (Powell, 1975), then 1D translates to 0.05% bp differences. Since in this study a D value of 0.1 corresponds to at least 1.5% single-copy divergence (the issue of the conversion of ΔT_m to percentage of base-pair mismatch will be discussed later), genetic divergence estimates based on isozymes are roughly 30 times smaller than

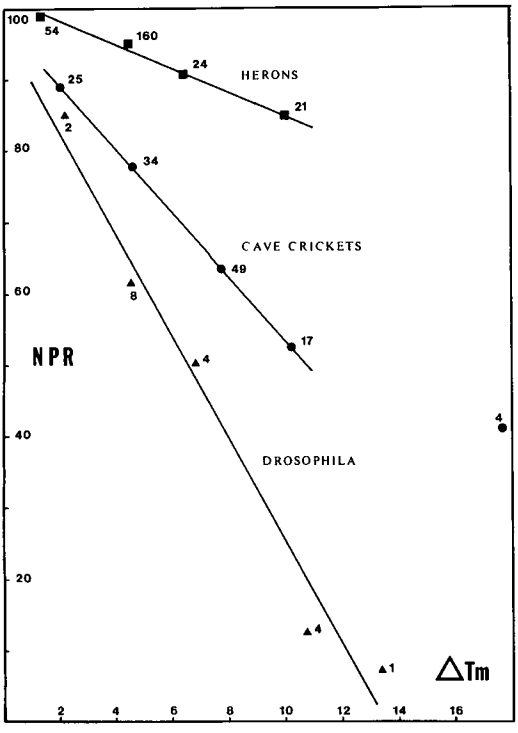

FIG. 3. Relationship between ΔT_m values (x-axis) and normalized percentage reassociation (NPR; y-axis) for cave crickets, *Drosophila* (Schulze and Lee, 1986), and herons (Sheldon, 1986). ΔT_m's were grouped into 3°C intervals: 1–3, 3–6, etc. The mean T_m and mean NPR for that interval are plotted. Numbers near points refer to the number of values averaged for that interval.

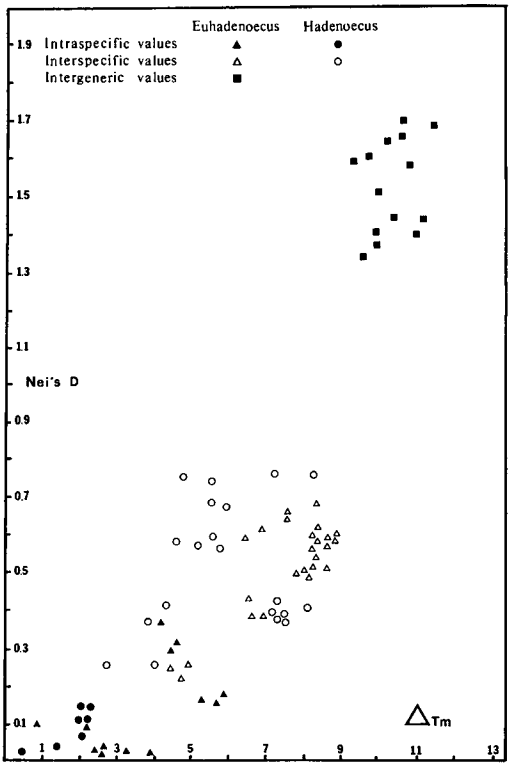

FIG. 4. Relationship between ΔT_m values (x-axis) and allozyme genetic distances (Nei, 1972) for 23 North American cave cricket populations.

estimates of divergence based on single-copy DNA sequence. Britten (1986*b*) has reached similar conclusions for sea urchins and *Drosophila*. Admittedly, these estimates are based on many assumptions. Nevertheless, the conclusion that coding sequences are conservative relative to total DNA is consistent with DNA sequence data (e.g., Coyne and Kreitman, 1986; Ashburner et al., 1984).

DISCUSSION

ΔT_m, *Percentage Mismatch, and NPR*

The first issue to be addressed is the conversion of ΔT_m to percentage of base-pair mismatch. Generally this conversion is taken to be 1:1, i.e., a ΔT_m of 1°C corresponds to 1% base-pair mismatch (Britten et al., 1974; Britten, 1986*a*). Recent data from this laboratory indicate that a ΔT_m of 1°C represents 1.5–2% mismatch (Powell et al., 1986; unpubl.). However, for the discussion here, we will adopt the more conservative 1:1 conversion, bearing in mind that the accuracy of this conversion is not settled.

Unlike some workers, we have chosen to present ΔT_m and the NPR values separately. It is important to note that the T_m, as we use it, is different from what other workers have called "T median" (Britten, 1986*a*) or T_{50}H (Sibley and Ahlquist, 1983). Our T_m's are calculated as the median melting temperature of duplexes that are formed. The percentage of reassociation does not enter into the calculations; i.e., sequences that are so divergent that they do not form stable duplexes under the reassociation conditions (75% match required) are not considered. The T median and T_{50}H as used by Britten and by Sibley and Ahlquist combine the NPR and ΔT_m to obtain an estimate of the median melting temperature of all sequences, including those that did not reassociate. Clearly, then, our ΔT_m is a conservative measure of divergence compared to

measures based on the T median or $T_{50}H$; ΔT_m is dependent on a relatively slowly evolving portion of the genome.

The conservativeness of ΔT_m is evident from Figure 3. The slope of the line for the cricket data indicates that for each degree increase in ΔT_m, the NPR drops by 4–5%. It is interesting to note that the linearity of the relationship drops off for distant comparisons, i.e., the comparisons of North American with European species. This implies that there may be a fraction of the genome that is conserved; it may be the portion of the genome that makes a cricket a cricket. From Figure 3 this portion would appear to be around 40%. However, this interpretation of the data should be taken with caution, as the 40% that is still hybridizing in this case has a ΔT_m of 18°C, which means it is approaching the point where it would no longer hybridize under the stringency used.

There are two, not mutually exclusive, explanations for the decrease in NPR. One is that there is a fraction of the genome that is highly variable and evolving rapidly. Alternatively, the lower NPR may be due to insertion/deletion differences between tracer and driver. The asymmetry in NPR for reciprocal hybridizations discussed in the Results indicates that there are fairly large genome-size differences among these taxa, even among populations of the same species!

It is also of interest to note that the drop in NPR for a given ΔT_m is greater for insects than for birds (Fig. 3). *Drosophila* have the fastest rate of decrease: an increase of 1°C ΔT_m is accompanied by a 7% decrease in NPR. For birds, a ΔT_m of 1°C is associated with only 1–2% decrease in NPR. Thus, not only is the rate of evolution of DNA different in different taxa (Britten, 1986*a*), but even the dynamics or mechanisms of change are different. These differences may be due to the relative rates of point mutations (reflected primarily in ΔT_m) and rates of insertion/deletions, changes in genome sizes, and/or proportion of the genome that is free to be highly variable (reflected primarily in decreased NPR).

Phylogenies and Rates

With a few exceptions, the pattern of branching derived from DNA-DNA hybridization data (Fig. 2) agrees with that obtained by isozyme analysis (Caccone and Sbordoni, 1987 fig. 3) and, to a lesser degree, with that obtained from morphological considerations (Hubbell and Norton, 1978). For the genus *Euhadenoecus,* all three approaches arrive at the same conclusion: the two forest-dwelling species (*E. adelphus* and *E. puteanus*) are most closely related, with *E. fragilis* clustering next and with *E. insolitus* the furthest diverged. In the case of *Hadenoecus,* the results of these approaches are not so congruent. Based on morphological and biogeographical considerations, Hubbell and Norton (1978) placed *H. cumberlandicus* and *H. subterraneus* in a clade separate from the other three species. Both the isozyme dendogram (Caccone and Sbordoni, 1987) and the ΔT_m dendogram (Fig. 2) separate *H. subterraneus* and place *H. cumberlandicus* with the other species. However, isozymes and ΔT_m disagree with respect to the placement of *H. jonesi.* The isozyme analysis placed *H. jonesi* in the *barri-opilionoides-cumberlandicus* clade, whereas ΔT_m values indicate that it is the most distant of all the species of the genus and that it separated first.

Which of the phylogenies is correct? While the technique of DNA-DNA hybridization has come under attack (Farris, 1985; Cracraft, 1985; Templeton, 1985) good counter arguments can be made for its power (Gould, 1985; Sibley and Ahlquist, 1983). Furthermore, the data presented here refute many of the criticisms of the technique: the repeatability of the technique is very good (note the small standard errors) and reciprocity (in the case of ΔT_m) is very good. T. H. Hubbell, the foremost systematist of these crickets, has agreed that the phylogeny indicated by the DNA-DNA hybridization data is compatible with morphological considerations (pers. comm.).

We have used the least-squares method (Felsenstein, 1984) to test whether the ΔT_m values used to construct the dendogram in Figure 2 change synchronously or asynchronously in the different branches, i.e., is there a clock? Because the data set is not a complete matrix, 14 subsets of the data which represent complete matrices were tested (see Results). All nodes and all branches were tested at least once. In no

case out of 14 tests did the asynchronous model give a significantly better fit than the synchronous model; thus, we cannot reject the clock hypothesis.

Given that the ΔT_m values behave as a clock, can we calibrate it with some absolute measure of time? Based on morphological, geological, and biogeographical considerations, Hubbell and Norton (1978) suggested that the two genera (*Euhadenoecus* and *Hadenoecus*) separated sometime in the Pliocene (2–7 MY ago) and that the speciation events within the genera occurred in the early Pleistocene (<2 MY ago). If this is true, these crickets are among the fastest evolving (with respect to DNA) organisms yet studied. The least related species within the genera have ΔT_m's of 7°C–8°C. Could this degree of divergence occur within 2 MY? It is difficult to assess the real significance of this, given that within-species divergence may be up to 5°C ΔT_m. Nevertheless, all indications are that cave crickets are rapidly evolving relative to birds and primates, for which a ΔT_m of 1°C has been estimated to correspond to 4–5 MY since the last common ancestor (Sibley and Ahlquist, 1984). Rates in invertebrates (*Drosophila* and sea urchins) have been estimated to be about five times this rate, i.e., a ΔT_m of 1°C indicates 0.5–1.5 MY of divergence (Britten, 1986*a*; Powell et al., 1986). Given the uncertainty of dating speciation events, the safest conclusion is that cave crickets are evolving relatively rapidly, at a rate that is probably similar to the rates in other invertebrates.

Taxonomic Considerations

Sibley and Ahlquist (1983) have amassed a very large data set of ΔT_{50}H's among thousands of species of birds. They have proposed that data from DNA-DNA hybridization experiments are quite consistent with taxonomic levels (Sibley and Ahlquist, 1983): ΔT_{50}H's between genera range from 0°C to 5°C; those between tribes range from 5°C to 7°C; those between families range from 9°C to 11°C; and those between superfamilies range from 13°C to 15°C. Obviously, such correspondence does not occur in cave crickets. Species of the same genus of cave crickets may differ by as large a ΔT_m as the ΔT_{50}H between families of birds. Recall that ΔT_m is a relatively conservative estimate of divergence compared to ΔT_{50}H. *Drosophila* are similar to crickets in this regard: ΔT_m's among species of this genus may be 10°C or more with very low NPR (Schulze and Lee, 1986; Zwiebel et al., 1982; Fig. 3).

Given this rapid rate of DNA divergence in invertebrates (i.e., cave crickets, *Drosophila,* and sea urchins), how useful can DNA-DNA hybridization be in reconstructing higher-category phylogenies? This will depend on the particular group being considered. For example, it is unlikely to be useful above the genus level with *Drosophila,* whereas with the present data, comparison of tribes was possible. However, it seems unlikely that it will be possible to extend the technique to levels that are possible with vertebrates. Measurement of ΔT_m above 15°–20°C becomes unreliable. Furthermore, considering the relatively rapid rate of decline of NPR with ΔT_m (Fig. 3), one begins to lose one of the advantages of the technique: average divergence at less and less of the total genome is being measured.

Isozymes Versus ΔT_m

As expected, there is a fairly good correlation between genetic distance as measured by isozyme electrophoresis and DNA-DNA hybridization (Fig. 4). However, the correlation is not perfect. This may lead to discrepancies in phylogenies based on the two sets of data (compare fig. 3 of Caccone and Sbordoni [1987] with Fig. 2). When such discrepancies arise, which is more reliable? For two reasons, we argue that the DNA-DNA hybridization data probably reflect the true phylogeny more reliably. First, differentiation over more of the genome is being measured. Second, the type of differentiation being measured by isozyme analysis (amino acid replacements) may be more subject to selection than are most base changes.

While in theory Nei's D and ΔT_m are measuring the same thing (genetic differentiation), they are based on different types of data. D is based on allele-frequency differences as determined by studies of individuals. ΔT_m, in the present case, is based on sequence divergence between DNA prepared from different populations. Thus, the

less-than-perfect correspondence of the two measures is not surprising. Another possible source of discrepancy between the two techniques might involve the average heterozygosity at isozyme loci and the T_m of DNA prepared from a population. The most extreme differences in isozyme heterozygosity in the present study are between either of the parthenogenetic populations (BEC and BAT) with low heterozygosity and all the rest (Caccone, 1986). Yet the T_m's for DNA from the parthenogenetic populations are not higher than those for the sexual populations (Appendix).

Conclusions

Several conclusions may be drawn from this study. 1) Cave cricket DNA is evolving at a very rapid rate, at least as rapid as in other invertebrates (i.e., *Drosophila* and sea urchins) and perhaps even faster. The ΔT_m as we use it is relatively conservative. There also appears to be rapid change in genome size, as reflected in the asymmetry of NPR's for reciprocal comparisons. 2) The phylogeny derived from DNA-DNA hybridization data generally agrees with that derived from morphology and isozymes. Where discrepancies arise, we argue the DNA data are probably more reliable. 3) To the best of our ability to test statistically for asynchrony, we could not reject the molecular-clock hypothesis. 4) Compared to vertebrates, high ΔT_m's occur at much lower taxonomic levels in cave crickets. Considerable variation exists even within species; in birds, this variation would be typical of different genera. 5) ΔT_m and Nei's D for isozymes are correlated, though not perfectly. For crickets the relationship is that a D of 0.1 corresponds to a ΔT_m of about 1.5°C. This indicates that protein-coding regions of the genome are evolving at a much slower rate than the average rate for total single-copy DNA.

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APPENDIX

In each table (A–X), all the experiments performed with a particular tracer are reported. The median melting temperature of the DNA duplexes is symbolized by t_m. TL is the mean tracer length in base pairs as determined by alkaline electrophoresis. T_m is the t_m corrected for tracer length; the correction is 500/TL (Hall et al., 1980). The absolute percentage of reassociation (R%) and the normalized percentage of reassociation relative to the homoduplex (NPR) are shown. N refers to the number of experiments, and SE is the standard error. For cases in which only two measurements were made, the ranges are reported in parentheses.

A) Tracer = HIG:

	Driver DNA									
	HIG (N = 3)	MON (N = 7)	HOR (N = 6)	SEN (N = 6)	MCL (N = 4)	NEW (N = 3)	ARG (N = 3)	IND (N = 4)	MAS (N = 4)	BAT (N = 3)
t_m (°C)	55.25	51.59	51.43	51.78	49.24	49.43	46.88	48.04	47.70	45.78
SE	0.09	0.11	0.08	0.06	0.18	0.16	0.13	0.15	0.26	0.04
TL (bp)	140	102	198	235	182	182	161	239	199	228
T_m (°C)	58.83	56.48	53.95	53.91	51.99	52.17	49.98	50.13	50.21	47.87
R%	75.5	68.8	58.1	62.7	50.2	49.3	45.5	45.4	45.9	41.9
NPR	100.0	91.1	77.0	83.1	66.6	65.3	60.3	60.2	60.9	55.5

B) Tracer = MON:

	Driver DNA		
	MON (N = 6)	HIG (N = 3)	ARG (N = 3)
t_m (°C)	57.96	55.41	49.24
SE	0.16	0.06	(0.22)
TL (bp)	416	313	304
T_m (°C)	59.16	57.00	50.88
R%	74.3	64.1	40.8
NPR	100.0	86.3	54.9

C) Tracer = HOR:

	Driver DNA				
	HOR (N = 9)	SEN (N = 3)	HIG (N = 4)	ARG (N = 5)	IND (N = 4)
t_m (°C)	57.01	54.87	49.04	47.59	45.74
SE	0.23	0.15	0.13	0.20	0.05
TL (bp)	270	372	93	149	118
T_m (°C)	58.86	56.21	54.43	50.95	49.99
R%	74.0	65.6	45.6	38.8	37.2
NPR	100.0	88.6	61.7	52.5	50.4

APPENDIX. Continued.

D) Tracer = SEN:

	Driver DNA					
	SEN (N = 5)	HOR (N = 3)	MON (N = 6)	NEW (N = 4)	IND (N = 3)	MAS (N = 4)
t_m (°C)	56.69	54.32	51.99	48.51	41.89	41.31
SE	0.18	0.20	0.15	0.01	0.08	0.09
TL (bp)	352	389	297	186	63.5	59.0
T_m (°C)	58.11	55.60	53.67	51.19	49.76	49.77
R%	70.0	61.7	54.9	36.6	43.1	45.5
NPR	100.0	88.2	78.4	52.3	61.6	65.0

E) Tracer = GIL:

	Driver DNA							
	GIL (N = 6)	NEW (N = 6)	MCL (N = 6)	LIP (N = 6)	SEN (N = 5)	ARG (N = 4)	HIS (N = 3)	GRE (N = 3)
t_m (°C)	56.91	56.13	53.34	52.66	50.08	48.07	46.73	46.86
SE	0.11	0.16	0.19	0.33	0.14	0.21	0.06	0.18
TL (bp)	416	414	547	425	334	259	367	398
T_m (°C)	58.11	57.34	54.45	53.86	51.58	49.75	48.09	48.20
R%	85.8	76.4	71.1	73.3	67.6	52.2	46.9	47.8
NPR	100.0	89.0	82.8	85.4	78.8	60.8	54.7	55.7

F) Tracer = LIP:

	Driver DNA			
	LIP (N = 3)	MCL (N = 9)	GIL (N = 3)	NEW (N = 5)
t_m (°C)	56.24	54.68	51.99	52.37
SE	0.14	0.19	0.08	0.32
TL (bp)	313	472	442	371
T_m (°C)	57.84	55.74	53.12	53.72
R%	75.6	69.4	57.2	57.5
NPR	100.0	91.8	75.6	76.0

APPENDIX. Continued.

G) Tracer = MCL:

	Driver DNA									
	MCL (N = 5)	LIP (N = 3)	GIL (N = 3)	NEW (N = 3)	HOR (N = 4)	SEN (N = 4)	HIG (N = 5)	IND (N = 4)	MAS (N = 4)	BAT (N = 4)
t_m (°C)	57.13	54.40	52.59	52.72	49.03	49.39	47.24	48.90	48.77	47.41
SE	0.16	0.13	0.20	0.14	0.26	0.11	0.20	0.07	0.22	0.09
TL (bp)	392	455	537	511	266	331	115	297	385	383
T_m (°C)	58.41	55.50	53.52	53.70	50.91	50.90	51.59	50.58	50.07	48.71
R%	80.3	77.4	77.2	77.6	65.8	67.9	65.6	59.8	60.1	51.8
NPR	100.0	96.4	96.1	96.6	82.0	84.5	81.7	74.5	74.8	64.5

H) Tracer = NEW:

	Driver DNA							
	NEW (N = 3)	GIL (N = 4)	MCL (N = 5)	LIP (N = 3)	HOR (N = 4)	HIG (N = 7)	IND (N = 4)	MAS (N = 5)
t_m (°C)	56.48	55.63	52.64	52.84	49.56	47.60	46.65	48.85
SE	0.23	0.08	0.18	0.07	0.26	0.10	0.03	0.06
TL (bp)	325	386	284	516	270	117	157	324
T_m (°C)	58.02	56.96	54.40	53.81	51.41	51.88	49.83	50.39
R%	73.6	55.9	52.3	57.6	41.9	45.6	42.2	46.6
NPR	100.0	75.9	71.0	78.3	56.8	61.9	57.3	63.2

I) Tracer = ARG:

	Driver DNA									
	ARG (N = 4)	BEC (N = 6)	IND (N = 6)	MAS (N = 9)	HOR (N = 4)	HIG (N = 4)	MON (N = 3)	MCL (N = 4)	BAT (N = 8)	BBC (N = 2)
t_m (°C)	56.23	51.58	51.62	51.69	46.77	43.45	46.63	48.01	47.60	45.26
SE	0.20	0.26	0.12	0.24	0.14	0.03	0.15	0.08	0.32	(0.06)
TL (bp)	278	516	389	430	185	83	160	285	437	218
T_m (°C)	58.03	52.55	52.90	52.85	49.47	49.48	49.74	49.76	48.74	47.58
R%	98.5	71.3	68.1	70.3	37.6	26.7	28.0	27.0	39.4	40.3
NPR	100.0	72.4	69.1	71.3	38.1	27.1	28.4	27.4	40.0	40.9

APPENDIX. Continued.

J) Tracer = BEC:

	Driver DNA			
	BEC ($N = 3$)	MAS ($N = 6$)	IND ($N = 3$)	ARG ($N = 3$)
t_m (°C)	56.53	55.66	54.20	52.22
SE	0.14	0.28	0.15	0.35
TL (bp)	156	429	407	488
T_m (°C)	59.73	56.79	55.43	53.24
R%	81.2	81.3	73.3	60.7
NPR	100.0	100.2	90.3	74.7

K) Tracer = IND:

	Driver DNA				
	IND ($N = 4$)	MAS ($N = 6$)	BEC ($N = 4$)	ARG ($N = 3$)	HIG ($N = 3$)
t_m (°C)	56.25	53.95	53.81	51.21	44.55
SE	0.25	0.23	0.38	0.04	0.03
TL (bp)	243	387	493	507	94
T_m (°C)	58.31	55.24	54.82	52.20	49.88
R%	85.6	74.9	68.3	63.2	41.1
NPR	100.0	87.4	79.8	73.8	48.0

L) Tracer = MAS:

	Driver DNA									
	MAS ($N = 4$)	IND ($N = 4$)	BEC ($N = 3$)	ARG ($N = 7$)	HOR ($N = 5$)	SEN ($N = 5$)	HIG ($N = 4$)	MON ($N = 4$)	MCL ($N = 3$)	NEW ($N = 4$)
t_m (°C)	56.29	53.40	55.48	51.50	47.74	47.92	45.15	47.04	48.06	47.96
SE	0.22	0.37	0.19	0.11	0.23	0.13	0.14	0.11	0.22	0.11
TL (bp)	207	318	434	426	182	187	102	166	180	216
T_m (°C)	58.71	54.97	56.63	52.67	50.49	50.59	50.05	50.06	50.84	50.26
R%	75.6	64.5	59.0	48.0	43.9	46.3	47.7	41.2	40.2	44.1
NPR	100.0	85.4	78.1	63.5	58.0	61.2	63.1	54.5	53.2	58.4

APPENDIX. Continued.

M) Tracer = CUM:

	Driver DNA					
	CUM (N = 3)	BBC (N = 3)	GRE (N = 6)	MAM (N = 3)	BAT (N = 3)	HIS (N = 2)
t_m (°C)	56.72	53.77	49.30	50.75	52.50	52.72
SE	0.22	0.10	0.33	0.22	0.23	(0.27)
TL (bp)	306	269	311	241	283	251
T_m (°C)	58.35	55.63	50.91	52.83	54.27	54.71
R%	88.7	77.0	77.4	73.8	76.6	71.2
NPR	100.0	86.8	87.3	83.2	86.3	80.3

N) Tracer = BAT:

	Driver DNA					
	BAT (N = 8)	WIN (N = 3)	HIS (N = 3)	MAM (N = 3)	BBC (N = 3)	DOL (N = 1)
t_m (°C)	57.00	52.43	55.36	50.38	52.61	37.95
SE	0.13	0.25	0.11	0.37	0.17	—
TL (bp)	341	140	292	295	316	159
T_m (°C)	58.46	56.01	57.07	52.08	54.19	41.08
R%	71.7	71.2	69.0	65.4	63.5	32.1
NPR	100.0	99.3	96.3	91.3	88.5	44.8

O) Tracer = HIS:

	Driver DNA												
	HIS (N = 5)	KOG (N = 5)	WIN (N = 3)	BAT (N = 4)	BBC (N = 4)	WHE (N = 3)	LIM (N = 3)	GRE (N = 3)	GIL (N = 3)	MCL (N = 3)	HIG (N = 3)	MON (N = 3)	ARG (N = 3)
t_m (°C)	57.70	53.84	54.60	56.48	53.57	52.40	49.19	50.15	47.97	46.65	46.52	45.90	45.71
SE	0.13	0.23	0.20	0.19	0.11	0.26	0.02	0.10	0.13	0.15	0.21	0.07	0.26
TL (bp)	470	228	284	543	398	370	391	579	552	341	211	299	276
T_m (°C)	58.76	56.03	56.36	57.40	54.83	53.75	50.47	51.01	48.88	48.12	48.89	47.57	47.55
R%	78.8	65.3	71.8	70.8	58.7	52.8	53.6	54.6	46.7	41.4	41.7	42.5	41.0
NPR	100.0	83.4	91.7	90.4	75.0	67.4	68.4	69.8	59.7	52.9	53.3	54.3	52.4

APPENDIX. Continued.

P) Tracer = KOG:

	Driver DNA		
	KOG ($N = 4$)	HIS ($N = 4$)	MAM ($N = 4$)
t_m (°C)	55.28	50.66	49.16
SE	0.28	0.06	0.12
TL (bp)	121	82	117
T_m (°C)	59.40	56.78	53.42
R%	73.2	58.9	53.2
NPR	100.0	80.4	72.7

Q) Tracer = WIN:

	Driver DNA			
	WIN ($N = 6$)	HIS ($N = 3$)	BAT ($N = 3$)	MON ($N = 2$)
t_m (°C)	57.89	56.35	55.67	46.84
SE	0.20	0.02	0.14	(0.02)
TL (bp)	355	494	338	230
T_m (°C)	59.30	57.36	57.15	49.01
R%	82.0	76.7	75.5	39.4
NPR	100.0	93.7	92.1	48.0

R) Tracer = GRE:

	Driver DNA							
	GRE ($N = 4$)	LIM ($N = 3$)	BBC ($N = 5$)	MAM ($N = 4$)	HIS ($N = 3$)	GIL ($N = 4$)	MON ($N = 6$)	ARG ($N = 4$)
t_m (°C)	58.18	57.65	50.65	50.29	50.91	47.68	47.98	45.20
SE	0.08	0.13	0.18	0.11	0.08	0.39	0.31	0.25
TL (bp)	502	490	327	271	454	350	300	146
T_m (°C)	59.18	58.67	52.18	52.13	52.01	49.11	49.65	48.60
R%	78.7	65.3	59.2	53.9	51.8	44.9	44.8	43.5
NPR	100.0	83.0	75.3	68.4	65.8	57.0	56.9	55.3

APPENDIX. Continued.

S) Tracer = LIM:

	Driver DNA			
	LIM (N = 3)	GRE (N = 3)	BBC (N = 3)	HIS (N = 3)
t_m (°C)	54.16	56.62	49.06	47.36
SE	0.20	0.06	0.07	0.25
TL (bp)	121	430	240	182
T_m (°C)	58.30	57.78	51.14	50.11
R%	72.1	64.3	58.1	48.5
NPR	100.0	89.2	80.5	67.3

T) Tracer = BBC:

	Driver DNA							
	BBC (N = 3)	BAT (N = 4)	HIS (N = 3)	GRE (N = 3)	LIM (N = 3)	MAM (N = 3)	WHE (N = 4)	ARG (N = 3)
t_m (°C)	58.24	53.69	53.47	49.73	49.76	51.70	52.26	46.76
SE	0.21	0.17	0.20	0.29	0.20	0.24	0.15	0.18
TL (bp)	890	681	312	327	546	281	379	225
T_m (°C)	58.76	54.42	55.07	51.26	50.68	53.48	53.58	48.98
R%	71.4	42.9	47.4	44.1	43.5	37.0	42.0	31.5
NPR	100.0	60.2	66.4	61.8	60.9	51.8	58.8	44.1

U) Tracer = BLF:

	Driver DNA		
	BLF (N = 3)	BBC (N = 3)	MAM (N = 2)
t_m (°C)	57.65	54.39	51.73
SE	0.21	0.05	(0.07)
TL (bp)	394	210	196
T_m (°C)	59.01	56.77	54.28
R%	70.4	66.4	55.3
NPR	100.0	94.4	78.6

APPENDIX. Continued.

V) Tracer = MAM:

	Driver DNA						
	MAM ($N = 3$)	WHE ($N = 3$)	BAT ($N = 4$)	BBC ($N = 4$)	GRE ($N = 4$)	LIM ($N = 3$)	ARG ($N = 3$)
t_m (°C)	57.45	55.10	51.61	51.57	49.93	49.64	43.68
SE	0.17	0.20	0.07	0.02	0.19	0.35	0.11
TL (bp)	243	216	224	212	279	338	99
T_m (°C)	59.50	57.40	53.84	53.92	51.72	51.12	48.74
R%	77.5	70.4	67.0	57.8	57.7	62.7	42.9
NPR	100.0	90.9	86.5	74.6	74.5	80.9	55.4

W) Tracer = WHE:

	Driver DNA			
	WHE ($N = 3$)	MAM ($N = 5$)	HIS ($N = 3$)	BBC ($N = 4$)
t_m (°C)	58.02	56.60	52.43	52.62
SE	0.18	0.17	0.12	0.17
TL (bp)	281	440	239	396
T_m (°C)	59.80	57.73	54.51	53.88
R%	74.9	74.5	64.3	66.1
NPR	100.0	99.5	85.9	88.3

X) Tracer = DOL:

	Driver DNA			
	DOL ($N = 3$)	MON ($N = 3$)	WIN ($N = 2$)	BAT ($N = 3$)
t_m (°C)	58.02	40.28	39.31	38.04
SE	0.20	0.25	(0.01)	0.51
TL (bp)	460	398	198	217
T_m (°C)	59.11	41.53	41.83	40.34
R%	80.5	37.4	30.5	30.7
NPR	100.0	46.4	37.9	38.1