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Molecular Phylogeny and Biogeography of *Kinosternon flavescens* Based on Complete Mitochondrial Control Region Sequences

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Nucleotide sequences for the complete mitochondrial control region (1158 bp) were used to determine the phylogenetic relationships among populations of the yellow mud turtle, *Kinosternon flavescens* (Kinosternidae). Phylogenetic analysis of the mtDNA sequences reveals a polyphyletic *K. flavescens* with three distinct clades: (1) *K. flavescens* of the Central Plains, including isolated populations of Illinois and Iowa, (2) *K. arizonense* in the Sonoran Desert of Arizona and Mexico, and (3) *K. durangoense* in the Chihuahuan Desert of Durango, Coahuila, and Chihuahua, Mexico. Sequence divergence and nucleotide diversity calculations support a hypothesis of Great Plains *K. flavescens* dispersal and subsequent isolation of populations in the Midwest related to climatic change during the Pleistocene. © 2001 Academic Press

Key Words: **Kinosternidae; *Kinosternon flavescens;* mtDNA; control region; Pliocene; Pleistocene.**

INTRODUCTION

The genetic and geographic structure of a species is dictated by current ecology and historical patterns of dispersal and vicariance. Intraspecific phylogenetics attempts to describe population structure and the processes of evolution responsible for this structure by assessing the degree of concordance between geographic location of haplotypes and their position in an inferred gene genealogy. Erroneous estimates of phylogeny may occur when morphological characters that represent phenotypic adaptations to specific environmental conditions and that do not reflect evolutionary history are used (Avise, 1986). For example, there have been three attempts to describe the geographic variation in the yellow mud turtle, *Kinosternon flavescens,* but all have been limited by the small number of variable, quantitative, morphological characters possessed by the species (Iverson, 1979; Houseal *et al.,* 1982; Berry and Berry, 1984).

The yellow mud turtle is a small, semiaquatic turtle that is widely distributed from central Illinois west to Nebraska and south to Sonora and the state of Durango, Mexico (Iverson, 1979). Historically, four subspecies of *K. flavescens* have been recognized: *K. f. flavescens* (Agassiz, 1857), *K. f. arizonense* Gilmore (1922), *K. f. spooneri* Smith (1951), and *K. f. durangoense* Iverson (1979). These designations were based, in part, on the disjunct distribution of *K. flavescens* (Iverson, 1979; Fig. 1). The species typically is found in well-draining soils of desert and grassland habitats. Individual *K. flavescens* exhibit high fidelity to migration paths between feeding ponds and estivation/hibernation sites (Iverson, 1991a). Life history characteristics such as site fidelity and low vagility, enforced by drainage limits and a disjunct distribution, allow the prediction that differentiation among populations of *K. flavescens* will be correlated with geographic distance.

Previous systematic studies of *K. flavescens* focused primarily on skin or shell coloration and ratios of shell measurement characters (Iverson, 1979; Houseal *et al.,* 1982; Berry and Berry, 1984). These characters allowed the recognition of the subspecies *K. f. durangoense* and supported the distinctiveness of *K. f. arizonense* (Iverson, 1979), but also resulted in differing conclusions regarding the taxonomic status of *K. f. spooneri* (Iverson, 1979; Houseal *et al.,* 1982; Berry and Berry, 1984). Conflicting results from previous studies indicate that additional data are needed to address intraspecific relationships among populations of *K. flavescens.* Analysis of mtDNA haplotypes offers an opportunity to examine the phylogenetic component of intraspecific differentiation of a species with limited morphological variation. Such a molecular data set would also be applicable to a phylogenetic analysis, rather than being limited to the phenetic methodologies used previously.

The goal of this study was to develop a phylogenetic hypothesis for *K. flavescens* populations, addressing questions of subspecific status and evidence for vicari-

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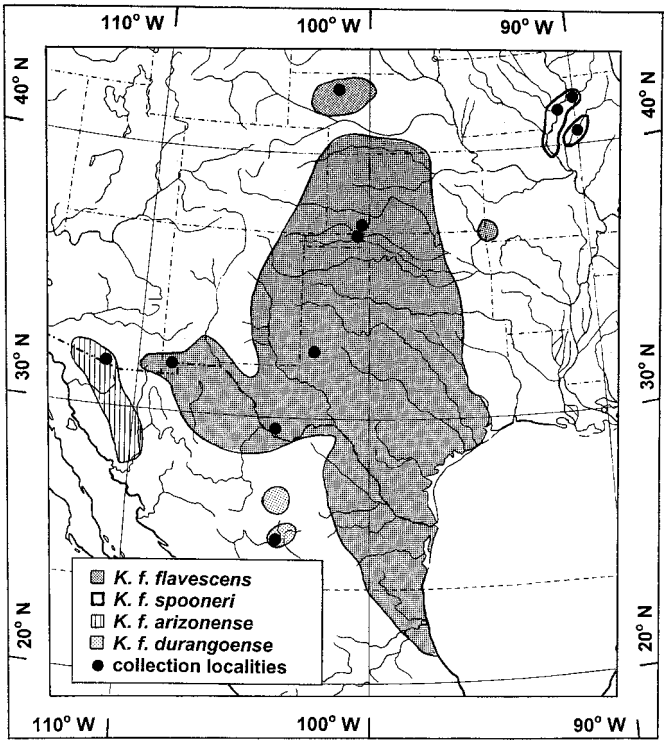

FIG. 1. Distribution map of the four currently recognized subspecies of *Kinosternon flavescens*. Location of specimens examined are indicated by dots.

ant or dispersal events using a molecular data set. For example, disjunct populations of *K. flavescens* in Illinois, Iowa, and Missouri may represent the dispersal of individuals from the Great Plains during extension of the prairie into the Midwest during the Xerothermic Interval of the Pleistocene (Smith, 1957). These populations then may have been isolated in well-drained, sandy soil habitats after a climatic shift resulting in a mesic environment occurred. In areas where geographic isolation mechanisms may not be as evident, congruent biogeographic patterns among unrelated groups can provide evidence for a broad vicariant event (Cracraft, 1982; Avise and Ball, 1990). Although there has not been a proposed mechanism that isolated the Sonoran and the Chihuahuan desert populations of *K. flavescens*, examination of phylogenetic relationship and geological history may provide an explanation for the observed pattern of endemism.

Because previous genetic studies have indicated a slower evolutionary rate in turtle mtDNA than in that of other vertebrates (Avise *et al.*, 1992), the mitochondrial control region (CR) was chosen based on the rapid evolutionary rate reported for this gene (Upholt and Dawid, 1977; Aquadro and Greenberg, 1983; Chang and Clayton, 1985). Because the control region accumulates base substitutions, additions, and deletions quickly, it has been used successfully for phylogenetic analysis of species with low genetic variability due to recent divergence or reduced microevolutionary rate (Avise *et al.*, 1992; Bernatchez *et al.*, 1992; Baker and Marshall, 1997; Good *et al.*, 1997; Lamb and Osentoski, 1997; Parker and Kornfield, 1997; Walker *et al.*, 1998).

Phylogenetic studies of turtles using control region sequences have been restricted to the 5′ end adjacent to the proline tRNA (Lamb *et al.*, 1994; Osentoski and Lamb, 1995; Encalada *et al.*, 1996; Lamb and Osentoski, 1997; Walker *et al.*, 1998, 1995; Walker and Avise, 1998; Roman *et al.*, 1999) This portion of the control region has provided some resolution in terms of genotypic variants and intraspecific phylogeography (Encalada *et al.*, 1996) and species/clade identification (Lamb *et al.*, 1994). Herein, we present complete control region sequences that we believe strengthen the argument for position homology and increase the number of informative characters in the data set.

MATERIALS AND METHODS

The sampling scheme was designed to assay intraspecific variability across the wide range of *K. flavescens*, including all subspecies and extant isolated populations (Table 1, Fig. 1). Seven populations of *K. f. flavescens* and three populations of *K. f. spooneri* were represented. Two individuals from each of the narrowly distributed subspecies, *K. f. arizonense* and *K. f. durangoense*, were also included. *Kinosternon baurii*, *K. hirtipes*, *K. subrubrum*, and *Sternotherus odoratus* were chosen as outgroup taxa in the phylogenetic analysis based on hypotheses of molecular (Seidel *et al.*, 1986; Starkey, 1997; Iverson, 1998) and morphological (Iverson, 1991b) character analyses of the Kinosternidae.

Total DNA was extracted from frozen whole blood, packed blood cells separated from plasma, or muscle and liver tissues. Blood samples from state-protected *K. f. spooneri* were collected from the dorsal cervical sinus as described by Bennett (1986). Tissues were homogenized on ice in STE buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA) and digested at 50°C after the addition of 50 μl of 20% SDS and 20 μl of 10 mg/ml proteinase K. The supernatant was extracted with buffered phenol (pH 8.0) and chloroform/isoamyl (24:1). Nucleic acids were precipitated overnight (−20°C) with 50 μl of 3 M NaOAc and 1.0 ml of absolute ethanol. For problematic blood samples with low DNA yields, an alternative isolation protocol was used (Bickham *et al.*, 1996).

An 1170-bp region containing the complete control region and flanking proline and phenylalanine tRNAs was amplified as a single fragment in a 50-μl polymerase chain reaction (PCR) using primers ChelProF (5′-CCGGTCCCCAAAACCGGAAC-3′) designed by Allard *et al.* (1994) and 12S-57R (5′-GATACTTGCATGTGTAAGTTT-3′) located within the conserved 5′ end of the

TABLE 1

Specimens Examined

Specimen		Locality	Museum Accession No.	GenBank Accession No.
K. f. arizonense	AZ1	Sasabe, Pima Co., AZ	JBI[a]	AF 316121
	AZ2	Sasabe, Pima Co., AZ	PCHP[b] 3756	AF 316122
K. f. durangoense	MX1	Pedriceña, Durango, Mexico	UF[c] 108889	AF 316124
	MX2	Pedriceña, Durango, Mexico	UF 108890	AF 316125
K. f. flavescens	NM	East of Playas, Hidalgo, Co., NM	JBI	AF 316129
	NE1	Three-corner Lake, Cherry Co., NE	UF 99882	AF 316127
	NE2	Three-corner Lake, Cherry Co., NE	UF 99883	AF 316128
	KS	Crooked River basin, Meade Co., KS	UF 80995	AF 316126
	OK	West of Forgan, Beaver Co., OK	UF 80996	AF 316130
	TX1	Linder Lake, Dawson Co., TX	TCWC[d] 68627	AF 316131
	TX2	Las Palomas WMA, Presidio Co., TX	TCWC 68625	AF 316132
K. f. spooneri	IA	Spring Lake, Muscatine Co., IA	JLC[e] 5999	AF 316133
	IL1	Mineral Marsh (Mississippi River), Henry Co., IL	No voucher taken	AF 316134
	IL2	Fornoff Pond (Illinois River), Mason Co., IL	No voucher taken	AF 316135
K. baurii		Crystal River, Citrus Co., FL	UF 80829	AF 316123
K. hirtipes		Río La Sauceda, Durango, Mexico	JBI	AF 316136
K. subrubrum		Goose Pond, Santa Rosa Co., FL	UF 80820	AF 316137
S. odoratus		Strong River, Simpson Co., MS	INHS[f] 12527	AF 316138

[a] JBI, John B. Iverson personal collection, Richmond, IN.
[b] PCHP, Peter C. H. Pritchard personal collection, Oviedo, FL.
[c] UF, Florida Museum of Natural History, University of Florida.
[d] TCWC, Texas Cooperative Wildlife Collection, Texas A&M University.
[e] JLC, John L. Christiansen personal collection, Des Moines, IA.
[f] INHS, Illinois Natural History Survey.

12S rRNA. The latter primer was designed by aligning sequences from *Homo*, *Xenopus*, *Trachemys*, and multiple fish taxa available on GenBank. Double-stranded PCRs contained 2.5 mM $MgCl_2$, 0.25 mM each deoxynucleotide, 0.5 mM each primer, and 2.5 units *Thermus aquaticus* DNA polymerase (Promega). Double-stranded amplification used the following conditions: initial denaturation for 5 min at 94°C and then 30 cycles of denaturation (94°C, 30 s), annealing (45–53°C, 30 s), and extension (72°C, 90 s). PCR products were run on 2% low-melt (65°C) NuSieve GTG agarose (BMA BioProducts) in 1× TAE running buffer. The target fragment was gel-isolated and purified following the protocol for Wizard PCR preps DNA purification system (Promega). PCR products that were not gel-isolated were purified using spin filtration columns (Millipore ultra-free-mc No. UFC3 LTK00).

Purified PCR products were ligated into pGEM-T vector plasmids (Promega) and transformed into DH5 alpha-*Escherichia coli* competent cells. Plasmids from positive colonies were isolated by alkaline lysis (PerfectPrep kit; 5 Prime-3 Prime Inc.) and used as templates in DNA sequencing reactions. The forward and reverse strands were sequenced from two separate clones for each specimen. A third clone was used to resolve any ambiguities. Chain-termination cycle sequencing was performed using the Delta-*Taq* Cycle Sequencing Kit (Amersham United States Biochemical, Cleveland, OH) or the fmol DNA Sequencing Kit (Promega). Internal primers were designed from sequence alignments (Table 2). Sequencing products were separated by electrophoresis through 6% polyacrylamide/8.3 M urea gels (SequaGel Sequencing System, National Diagnostics) and visualized by autoradiography.

Autoradiographs were read manually as data files in the program EasyReader (Hitachi Software Engineering). Complete double-stranded sequences were then assembled by overlapping sequence files in Sequencher (Gene Codes, Ann Arbor, MI). Nucleotide sequence data for 14 *K. flavescens* and the 4 outgroup taxa were aligned in Clustal W (Thompson *et al.*, 1994). The control region alignment (including gaps) of 18 taxa allowed the identification of some conserved elements: F-box, CSB1, and three domains of the control region

TABLE 2

Internal Sequencing Primers Used in This Study

Name[a]	Sequence	Strand
KNCR 271F	5′-ATCGTTATACATGGTTATCTATT-3′	Heavy
KNCR 562F	5′-GGTCTTACTTGCATATCGTAG-3′	Heavy
KNCR 581R	5′-CTACGATATGCAAGTAAGACC-3′	Light
KNPH 35R	5′-GCCGTGCTTTGATATAAGCT-3′	Light

[a] CR and PH indicate the control region and the phenylalanine tRNA genes, respectively. Numbers indicate base position in each of those genes, based on the 1170-bp amplified portion.

that have been found among highly divergent taxa (Southern *et al.*, 1988; Saccone *et al.*, 1991; Baker and Marshall, 1997; Zink and Blackwell, 1998a) (see Appendix). Using the criteria of Zink and Blackwell (1998a), domains were defined by the positioning of the F-box and CSB1 and the degree of base pair conservation. Domain I begins at origin of the CR and is terminated at the beginning of the F-box. Domain II is the central conserved region. Domain III begins at the origin of the CSB1, terminates at the phenylalanine tRNA, and is the most variable region. Regions of ambiguity in Domain III were removed from the final analysis. NBRF/PIR files were imported into PAUP* vers. 4.0b2 (Swofford, 1998) for phylogenetic analyses.

Both character-state and distance-based methods were used to estimate phylogenetic relationships within *K. flavescens*. All maximum-parsimony (MP) analyses employed a branch and bound search of the equal weighted data, and character states inferred as gaps were treated as missing data. Only minimum-length trees were retained and zero-length branches were collapsed. Bootstrap support for each node was determined using 2000 replications of a fast heuristic search available in PAUP*. Robustness of clades was also assessed using decay index values (Bremer, 1994), which calculate the number of additional steps needed to collapse nodes in the minimum-length tree using AutoDecay vers. 3.0 (Eriksson and Wikstrom, 1995). Phylogenetic signal was assessed in the aligned control region data set using the g_1 statistic (Hillis and Huelsenbeck, 1992). Tests for phylogenetic signal were conducted using the complete control region and each of the three domains separately. Phylogenetic hypotheses were also generated via the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987), using the Kimura two-parameter distance correction (Kimura, 1980). Alternative topologies to test the monophyly of *K. flavescens* were constructed as constraint trees in MacClade vers. 3.07 (Madison and Madison, 1992). The modified Templeton test (Templeton, 1983a,b; Felsenstein, 1985), which is based on the Wilcoxon sign-ranks test, was used to examine the statistical significance of the shortest tree length (most-parsimonious tree) relative to the alternative hypotheses. Tests were conducted in PAUP*, which incorporates a correction for tied ranks.

An effort was also made to compare *K. flavescens* sequences from this study to other kinosternid taxa. Although sequence data from *Sternotherus minor* and *S. odoratus* were unavailable on GenBank, we were able to compare *K. flavescens* to these taxa using Nei's equation 10.4 (Nei, 1987) of nucleotide (nt) diversity. Nt diversity has been calculated for *S. minor* and *S. odoratus* from partial control region sequence data (Walker *et al.*, 1995) and restriction site analysis of mtDNA (Walker *et al.*, 1997), respectively. By reducing the *K. flavescens* sequence data to the first 432 bp (= reduced data set), we were able to calculate nt diversity from the pairwise comparisons (Table 4) and compare it to Walker *et al.*'s estimations. Nt diversity was calculated for the *K. flavescens* species group and subgroups as defined by the recovered clades in MP and NJ analyses and current taxonomy.

RESULTS

The complete control region alignment for all 18 specimens contained 1158 base positions and exhibited 224 variable sites, 113 of which were phylogenetically informative under maximum-parsimony criteria. All mitochondrial control region sequences were aligned unambiguously for Domains I and II, with only a few single-base insertion/deletion events (indels) required. In Domain III, indels were more frequent and of varying lengths in relation to the AT repeat. The largest insertion/deletion event occurred in Domain III of the *S. odoratus* sequence, where a 22-bp insertion began at position 884, upstream from the AT repeat, and was succeeded by two shorter, nonrepetitive insertions. Ambiguous alignment of the large AT repeat (base positions 957–1034) required that we omit it from the analysis. This omission resulted in a data set consisting of 1082 base positions and exhibiting 193 variable sites, 97 of which were phylogenetically informative. The omission did not change the resulting topologies. Strong phylogenetic signal was detected for the control region data set (g_1 statistic $= -0.796$; $P < 0.01$).

Parsimony analysis for 18 taxa yielded 16 most-parsimonious trees of 269 steps, with a consistency index excluding uninformative characters of 0.682 (Fig. 2). Preliminary parsimony analyses had shown that the ingroup was nonmonophyletic with respect to the outgroup taxa, *K. baurii*, *K. hirtipes*, *K. subrubrum*, and *S. odoratus*. In subsequent analyses we designated *S. odoratus* as the outgroup and *K. baurii*, *K. hirtipes*, and *K. subrubrum* as functional outgroups based on previous molecular hypotheses of the Kinosternidae (Starkey, 1997; Iverson, 1998). *K. flavescens* was determined to be polyphyletic using the algorithm suggested by Farris (1974). Populations of *K. flavescens* formed two main clades, *K. f. flavescens* + *K. f. spooneri* and *K. f. arizonense* + *K. f. durangoense*. A clade composed of *K. baurii* and *K. subrubrum* was the sister group to the *K. f. flavescens* + *K. f. spooneri* clade. Variation among the 16 most-parsimonious tree topologies was restricted to the placement of the southwestern United States (New Mexico and Texas) populations of *K. f. flavescens* and relationships among the Great Plains/Midwest populations of *K. f. flavescens* and *K. f. spooneri*. Decay index values for internal branches ranged from 0 to 12 steps (Fig. 2).

The distance analysis using the NJ algorithm with the Kimura two-parameter distance measure resulted in a topology similar to that shown by MP (Fig. 3). Groups supported by bootstrap values >95% on the distance tree included all groups recovered as >95% in

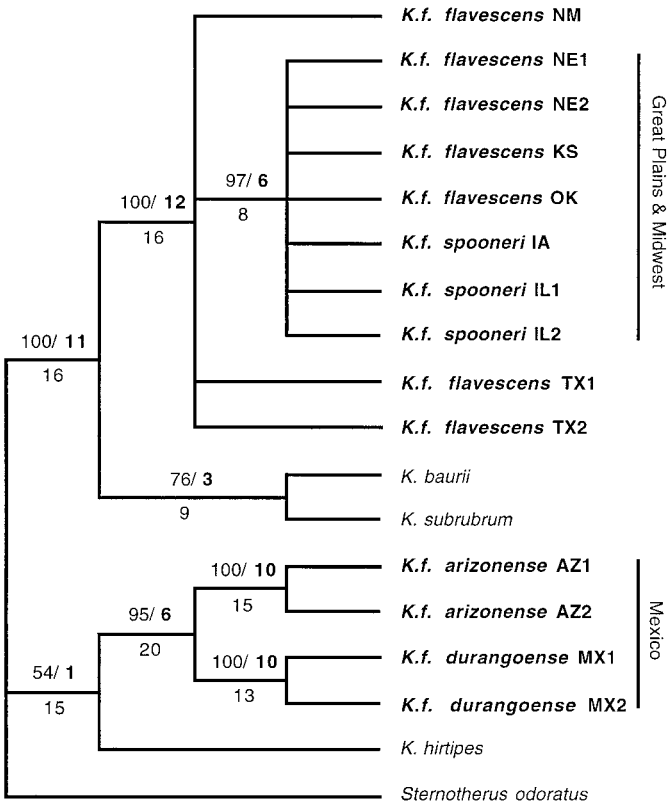

FIG. 2. Strict consensus tree of the 16 most-parsimonious trees (TL = 269; C.I. = 0.799; R.C. = 0.682) recovered in maximum-parsimony analysis of control region (omitting AT repeat). Bootstrap (2000 replications) and decay index values (in boldface) are indicated above branches. Character support is given below branches.

the MP analysis. The only exception was support in the NJ topology of a southwestern (New Mexico + Texas) *K. f. flavescens* clade.

To examine the nonmonophyly of *K. flavescens,* an alternative phylogenetic hypothesis was constructed in MacClade (Maddison and Maddison, 1992) by constraining the monophyly of the previously recognized *K. flavescens.* The new constraint topology was 18 steps longer and significantly different ($P < 0.0015$) from that of the most-parsimonious trees, which supports a nonmonophyletic *K. flavescens.*

Significant phylogenetic signal was detected in all three control region domains using 10^5 randomly generated trees (Table 3). Domain I contained 303 base positions with 68 variable positions; 42 were phylogenetically informative (g_1 statistic $= -0.775$; $P < 0.01$). Domain II contained 355 base positions with 40 variable sites; 16 were phylogenetically informative (g_1 statistic $= -0.952$; $P < 0.01$). Domain III contained 500 base positions with 116 variable positions; 55 were phylogenetically informative (g_1 statistic $= -0.711$; $P < 0.01$).

Within the currently recognized *K. flavescens,* nt diversity was widely variable (Tables 4 and 5). Pairwise nt diversity among all *K. flavescens* averaged 0.039 (3.9%), ranging from 0 to 0.085 (8.5%). When excluding *K. f. arizonense* and *K. f. durangoense,* the nt diversity was drastically reduced to 0.006 (less than 1%). Average nt diversity between *K. f. spooneri* and the nominate subspecies was 0.005 (0.5%). Average nt diversity between *K. f. arizonense* and *K. f. durangoense* was 0.015 (1.5%), much higher than that seen within *K. f. flavescens* + *K. f. spooneri.* The comparison between *K. f. flavescens* + *K. f. spooneri* and the Mexican subspecies averaged 0.080 (8.0%).

DISCUSSION

Significant phylogenetic signal was found in all three domains of the control region using the g_1 statistic, suggesting that Domain III may be as important in generating an accurate phylogeny of closely related taxa as the more commonly used Domains I and II

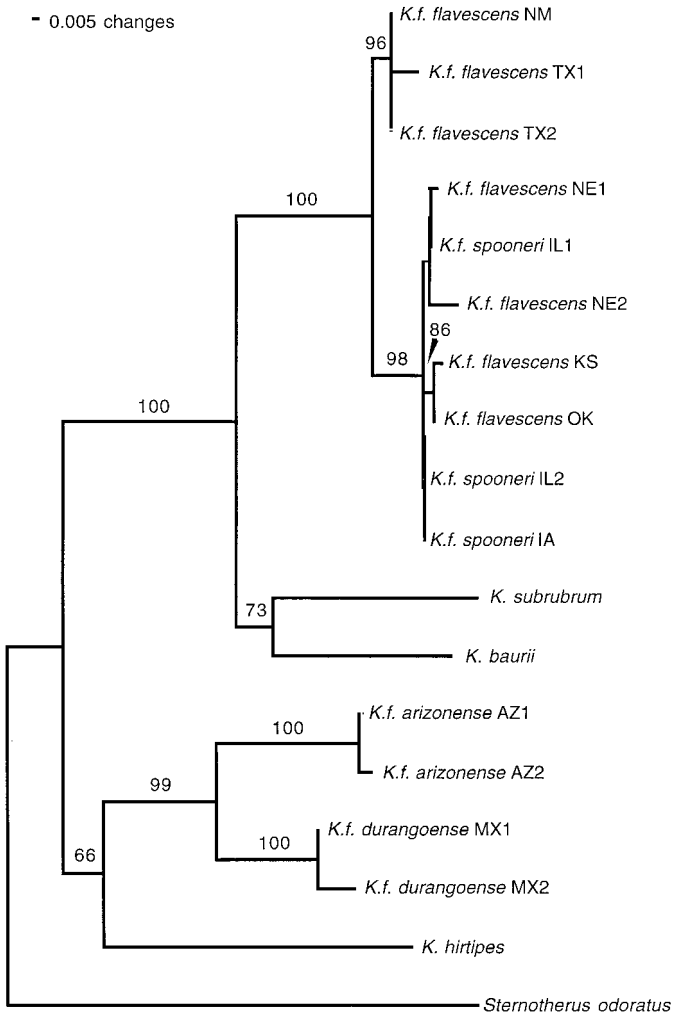

FIG. 3. Neighbor-joining tree using the Kimura two-parameter distance correction. Bootstrap values (>50%) from 2000 replications are indicated above branches.

TABLE 3

Distribution of Phylogenetically Informative and Variable Positions in the Complete CR and Its Three Domains

	g_1	Significance	Total positions	Variable positions	Phylogenetically informative sites
Complete CR[a]	−0.796	$P < 0.01$	1080	193	97
Domain I	−0.775	$P < 0.01$	303	68	42
Domain II	−0.952	$P < 0.01$	355	40	16
Domain III	−0.711	$P < 0.01$	500	116	55

[a] Omitting AT repeat (957–1036 bp).

(Table 3). For turtles, phylogenetic analyses have been performed using only the 5′ end (Domain I and partial Domain II) of the control region (Encalada *et al.*, 1996; Lamb *et al.*, 1997, 1994; Walker *et al.*, 1998, 1995; Roman *et al.*, 1999). It should be noted that when our data set was limited to this portion, some relationships were altered and overall branch support was reduced (J. M. Serb, unpublished). Although it is intuitive that a complete gene will generally provide more phylogenetically informative characters than a partial gene, we suggest that the potential utility of the control region has not been fully exploited.

Low Nucleotide Diversity among Central Plains K. flavescens Populations

Central Plains populations (*K. f. flavescens* + *K. f. spooneri*) have greatly reduced nt diversity (0.06%) compared to *S. minor* (1.7%) or *S. odoratus* (1.6%) (Walker *et al.*, 1995, 1997; respectively). The low nt diversity recovered among Central Plains populations can be explained by the species' natural history. *K. flavescens* inhabits xeric environments with unstable conditions, is more terrestrial (Iverson, 1991a), often migrating between bodies of water (Cope, 1892), and tends to have a large activity range (Mahmoud, 1969) compared to other kinosternid species, such as *S. odoratus*, which is more restricted to aquatic environments. In addition, genetic differentiation among populations will be slowed considerably by migration, and even infrequent migration is enough to prevent any appreciable genetic differentiation unless strong differential selection exists (Eq. 9.65 in Nei, 1987). An increase in the probability of contact among populations resulting from large activity ranges and dispersal due to unpredictable environmental conditions supports the molecular findings of relatively low nt divergence in Central Plains *K. flavescens*, even compared to the widely distributed species, *S. odoratus* (Walker *et al.*, 1997).

Pleistocene Isolation of K. f. spooneri Populations

Nucleotide diversity was also extremely low between the isolated *K. f. spooneri* populations and the nominate subspecies (Table 5). Although the sample size was small, six individuals (all *K. f. spooneri* and *K. f. flavescens* NE1, Kansas and Oklahoma) possessed the same haplotype, suggesting that the time of isolation

TABLE 4

Pairwise Comparison of Uncorrected Distance Among *Kinosternon flavescens* Taxa Using the Complete Control Region (above Diagonal) and Reduced CR (=432 bp) (below Diagonal) Data Sets

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	KflaNM	—	0.009	0.011	0.009	0.008	0.004	0.000	0.007	0.008	0.007	0.068	0.070	0.066	0.070
2	KflaNE1	0.012	—	0.004	0.004	0.003	0.013	0.009	0.002	0.001	0.002	0.070	0.072	0.067	0.069
3	KflaNE2	0.014	0.002	—	0.006	0.005	0.015	0.011	0.004	0.004	0.004	0.073	0.074	0.068	0.073
4	KflaKS	0.012	0.000	0.002	—	0.009	0.013	0.009	0.002	0.003	0.002	0.073	0.075	0.069	0.073
5	KflaOK	0.012	0.000	0.002	0.000	—	0.012	0.008	0.001	0.002	0.001	0.073	0.074	0.069	0.072
6	KflaTX1	0.002	0.014	0.016	0.014	0.014	—	0.004	0.011	0.012	0.011	0.073	0.074	0.071	0.073
7	KflaTX2	0.000	0.012	0.014	0.012	0.012	0.002	—	0.007	0.008	0.007	0.068	0.070	0.067	0.069
8	KspoIA	0.012	0.000	0.002	0.000	0.000	0.014	0.012	—	0.001	0.000	0.071	0.073	0.068	0.070
9	KspoIL1	0.012	0.000	0.002	0.000	0.000	0.014	0.012	0.000	—	0.001	0.071	0.073	0.067	0.070
10	KspoIL2	0.012	0.000	0.002	0.000	0.000	0.014	0.012	0.000	0.000	—	0.071	0.072	0.067	0.077
11	KariAZ1	0.073	0.078	0.080	0.078	0.078	0.075	0.073	0.078	0.078	0.078	—	0.001	0.026	0.030
12	KariAZ2	0.075	0.080	0.082	0.080	0.080	0.077	0.075	0.080	0.080	0.080	0.002	—	0.028	0.031
13	KdurMX1	0.080	0.080	0.082	0.080	0.080	0.082	0.080	0.080	0.080	0.080	0.019	0.021	—	0.006
14	KdurMX2	0.082	0.082	0.085	0.082	0.082	0.085	0.082	0.082	0.083	0.083	0.021	0.024	0.023	—

TABLE 5

Estimated Nucleotide Diversity (π^a) within Kinosternidae Taxa Using Reduced and Complete Data Sets of the Control Region

	Mean π (reduced CR)	Min	Max	Mean π (complete CR)
K. flavescens (all)	0.039	0	0.085	0.035
K. flavescens (Central Plains)	0.006	0	0.016	0.006
K. f. flavescens vs *K. f. spooneri*	0.005	0	0.014	0.005
K. flavescens (Central Plains) vs Mexican subspecies	0.080	0.073	0.085	0.071
K. f. arizonense vs *K. f. durangoense*	0.015	0.002	0.024	0.020
S. minor[b]	0.017	0.002	0.032	—
S. odoratus[c]	0.016	—	0.031	—

[a] Mean $\pi = [n(n-1)](\Sigma f_i f_j \pi_{ij})$, where f_1 and f_j are the frequencies of the ith and jth haplotypes in a sample size of n, and π_{ij} is the uncorrected estimate of sequence divergence between the ith and jth sequences (Nei, 1987).

[b] Walker *et al.* (1995).

[c] Walker *et al.* (1997).

between the Great Plains and the Midwest may have been too recent for fixed mutations to accumulate in the control region. Geological evidence supports a recent isolation event. Fossils of *K. flavescens* are known from the Hemphilian (Late Miocene/Early Pliocene) (Parmley, 1992) from Nebraska, which was not covered by the maximum extent of glaciation (Nebraskan and Kansan advances), unlike Iowa and the majority of Illinois. It has been hypothesized that these populations of *K. flavescens* persisted in a refugium in the Sand Hills of Nebraska during the glacial advances and became the founders of Midwestern populations during the expansion of the Prairie Peninsula.

A description of the postglacial climate change of the Midwest is given by Wright (1968). An accelerated climate change occurred 10,000–11,000 years ago as a result of the final retreat of the Wisconsinian ice sheet from the Great Lakes region. Boreal vegetation on the periphery of the retreating glacier was replaced by deciduous taxa that were gradually dominated by more xeric species (i.e., *Quercus* spp.) around 8000 years ago. By 7000 years ago, the Midwest had reached a maximum of warmth and dryness (Xerothermic Interval), allowing prairie grassland species from the west to dominate. After this peak of prairie expansion from the Great Plains into Illinois and Indiana (known as the Prairie Peninsula), a slow reversal in the climatic trend occurred. The climate cooled and annual precipitation increased more rapidly, creating a mesic environment by 4000 years ago. The xeric-adapted taxa that had invaded from the west during the Xerothermic Interval were either eliminated or restricted to well-drained, sandy soils (see Smith, 1957). Thus, the history of the Prairie Peninsula suggests that the time of isolation between *K. f. spooneri* and the nominate subspecies was only about 4000 years ago. The recent isolation event in conjunction with a seemingly slower evolutionary rate in turtle mtDNA (Avise *et al.*, 1992) would predict an unresolved polytomy and an undiagnosable *K. f. spooneri* subspecies, as was recovered in this study.

Differentiation of Mexican Subspecies

Molecular evidence strongly supports a distinct clade of *K. f. arizonense* and *K. f. durangoense.* These subspecies were separated from other *K. flavescens* by long branches, robust bootstrap support, relatively high decay index values, and many synapomorphies (Fig. 2). Indeed, the nt diversity between Mexican and Central Plains subspecies (7.3%) is more than four times the diversity calculated across populations of other wide-ranging kinosternids that have been studied (Table 5). These results clearly support our finding that *K. flavescens*, as previously recognized, is an unnatural group.

In addition to mitochondrial evidence, morphological and life history traits support the distinction of the Mexican subspecies. In previous morphometric analyses, *K. f. arizonense* and *K. f. durangoense* were consistently distinct from all other populations of *K. flavescens* and from each other (Iverson, 1979; Houseal *et al.*, 1982; Berry and Berry, 1984). Furthermore, morphological separation has been observed in skeletal to body mass ratios (Iverson, 1989). *K. f. arizonense* and *K. f. flavescens* also differ in numerous life history traits, including timing of the reproductive cycle, number of clutches per year, and the average percentage of gravid female body mass incorporated into the clutch of eggs (Iverson, 1989). Iverson (1989) has also shown that activity periods and reproductive cycles of *K. f. arizonense* are asynchronous with those of the nominate subspecies, and similar observations have been made for *K. f. durangoense* (J. B. Iverson, unpublished data). Environmental cues for activity periods also differ between *K. f. arizonense* and Central Plains *K. flavescens;* precipitation stimulates Mexican desert populations, whereas the gradual spring warming seems to be the stimulus for *K. f. flavescens* (Iverson, 1989). Incongruent activity periods suggest temporal as well as geographic isolation between Mexican and Central Plains populations.

Nucleotide diversity between *K. f. arizonense* and *K. f. durangoense* was estimated at 1.5%, higher than estimates across the range of Central Plains *K. flavescens* (Table 5). Geographically, the two Mexican taxa are isolated from each other by the Continental Divide, which also separates the closely related *K. hirtipes* and *K. sonoriense* (Iverson, 1981). Similar zoogeographic

patterns have been recovered in phylogenetically unrelated groups such as scaled quail (*Callipepla;* Zink and Blackwell, 1998b) and pocket mice (*Chaetodipus;* Riddle, 1995), supporting gene–geography concordance described by Cracraft (1982) and Avise and Ball (1990). Congruent zoogeographic patterns in the *K. flavescens* phylogeny, concordant with *Callipepla* and *Chaetodipus* phylogenies, implies an historical vicariant event which affected many different taxa.

The Continental Divide consists of the Sierra Madre Occidental and the Colorado Plateau, which forms a barrier between the Chihuahuan and the Sonoran deserts. Historically, the Cochise Filter Barrier, a 100- to 200-km-wide gap between these mountain ranges, has been the only physiographic portal along the western wall of the Chihuahuan desert (Morafka, 1977). Between events of orogenesis, this portal may have periodically provided an east–west route for gene flow between the two deserts during the Pliocene or Pleistocene (Morafka, 1977). Mammalian data (see Riddle, 1995) suggest that the isolation of the Sonoran and Chihuahuan deserts may have occurred over a single broad period or several events of the Late Miocene (Hemphillian) through the Pliocene (Blancan) during the secondary uplift of the Sierra Madre Occidental. Based on the fossil evidence of morphologically modern *K. f. arizonense* from the Late Pliocene (Blancan) (Gilmore, 1922), we favor either the secondary uplift of the modern Sierra Madre Occidental during the Miocene (Cohn, 1965) or the closing of the Cochise Filter Barrier during the Late Miocene/Early Pliocene as the event isolating the Sonoran and Chihuahuan *K. arizonense–K. durangoense* populations.

CONCLUSIONS: TAXONOMIC SUGGESTIONS FOR *K. flavescens* SUBSPECIES

Because sequences of the complete mitochondrial control region recovered a polyphyletic *K. flavescens*, the current taxonomic arrangement of its subspecies is not supported. We chose to recognize taxa using the monophyly version of the Phylogenetic Species Concept (de Queiroz and Donoghue, 1990). The molecular data support the recognition of three distinct species within *K. flavescens:* (1) *K. flavescens* of the Central Plains, including populations of the Illinois and Mississippi River drainages of Illinois, Iowa, and Missouri; (2) *K. arizonense* in the Sonoran Desert of Arizona and Mexico; and (3) *K. durangoense* in the Chihuahuan Desert of Durango, Coahuila, and Chihuahua, Mexico. Geographic isolation and differences in morphological and life history traits lend further support for these designations. Although the recognition of *K. f. spooneri* (as previously defined) is not supported using mitochondrial control region sequences, further study of phylogeography within *K. flavescens* (*sensu stricto*) may demonstrate variation that is worthy of subspecific designation. Finally, the phylogenetic placement of *K. arizonense* and *K. durangoense* within the Kinosternidae was not fully resolved in this study and will require further analysis including additional Mexican species. Once those phylogenetic relationships are clarified, hypotheses of speciation events can be investigated in a geological context.

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APPENDIX

Aligned Sequences of the Mitochondrial Control Region for All 18 Taxa

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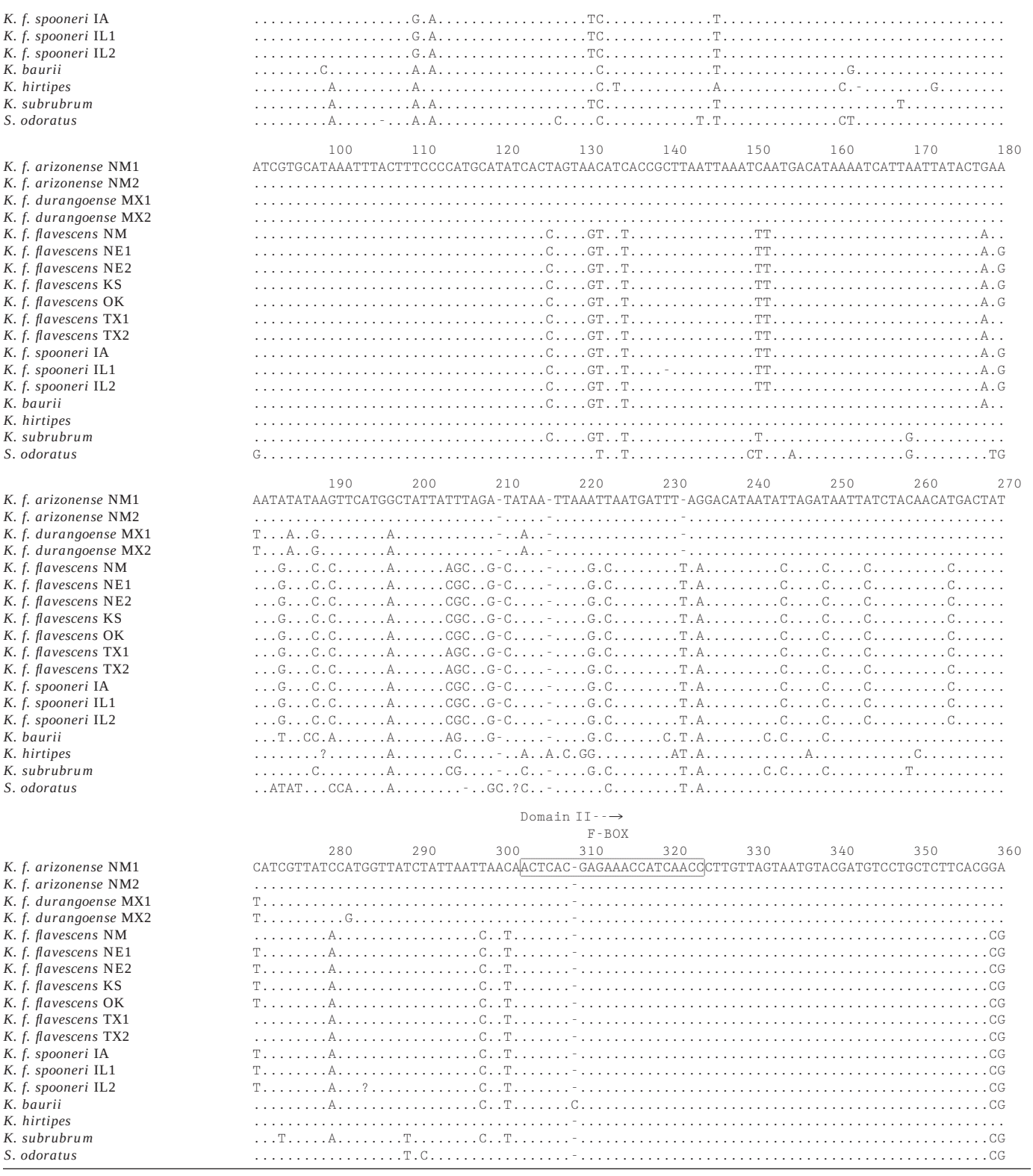
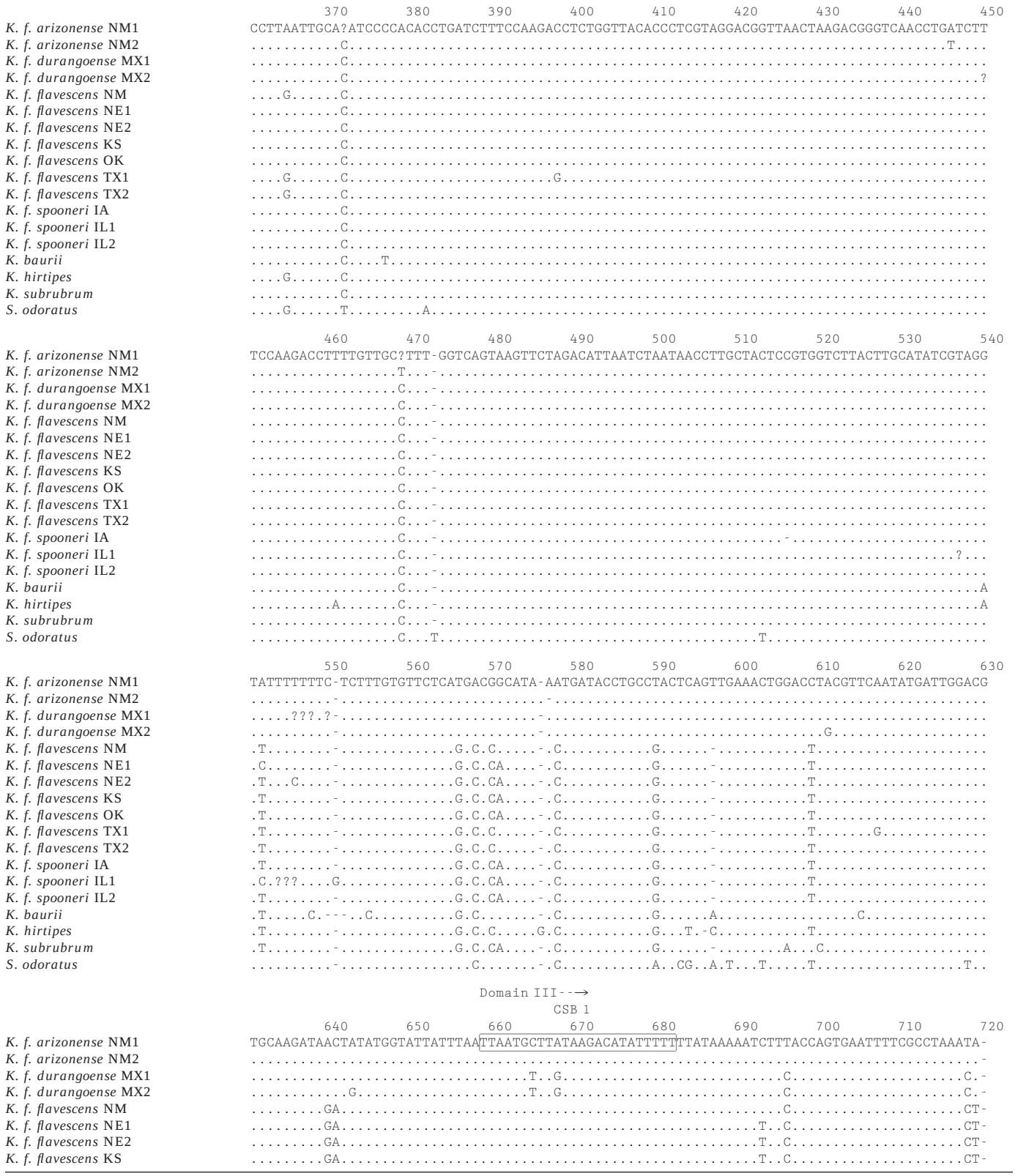
                          Domain I-->
                                10        20        30        40        50        60        70        80        90
K. f. arizonense NM1      ATAAAAGATGCGCATCTTTTCGCCATATTTTAGCCGTATTCTCCCTACCTAGTCACACTAGAGTAGTACCTAATATACACTCCTATGTAT
K. f. arizonense NM2      ..........................................................................................
K. f. durangoense MX1     ..................A.............C....C.....................................................
K. f. durangoense MX2     ..............?.....A.............C....C.....................................................
K. f. flavescens NM       .........A.........G.A.....................C.............T.....................................
K. f. flavescens NE1      ..................G.A.....................TC............T.....................................
K. f. flavescens NE2      ..................G.A.....................TC............T.....G...............................
K. f. flavescens KS       ..................G.A.....................TC............T.....................................
K. f. flavescens OK       ..................G.A.....................TC............T.....................................
K. f. flavescens TX1      .........A.........G.A.....................C.............T.....................................
K. f. flavescens TX2      .........A.........G.A.....................C.............T.....................................
```

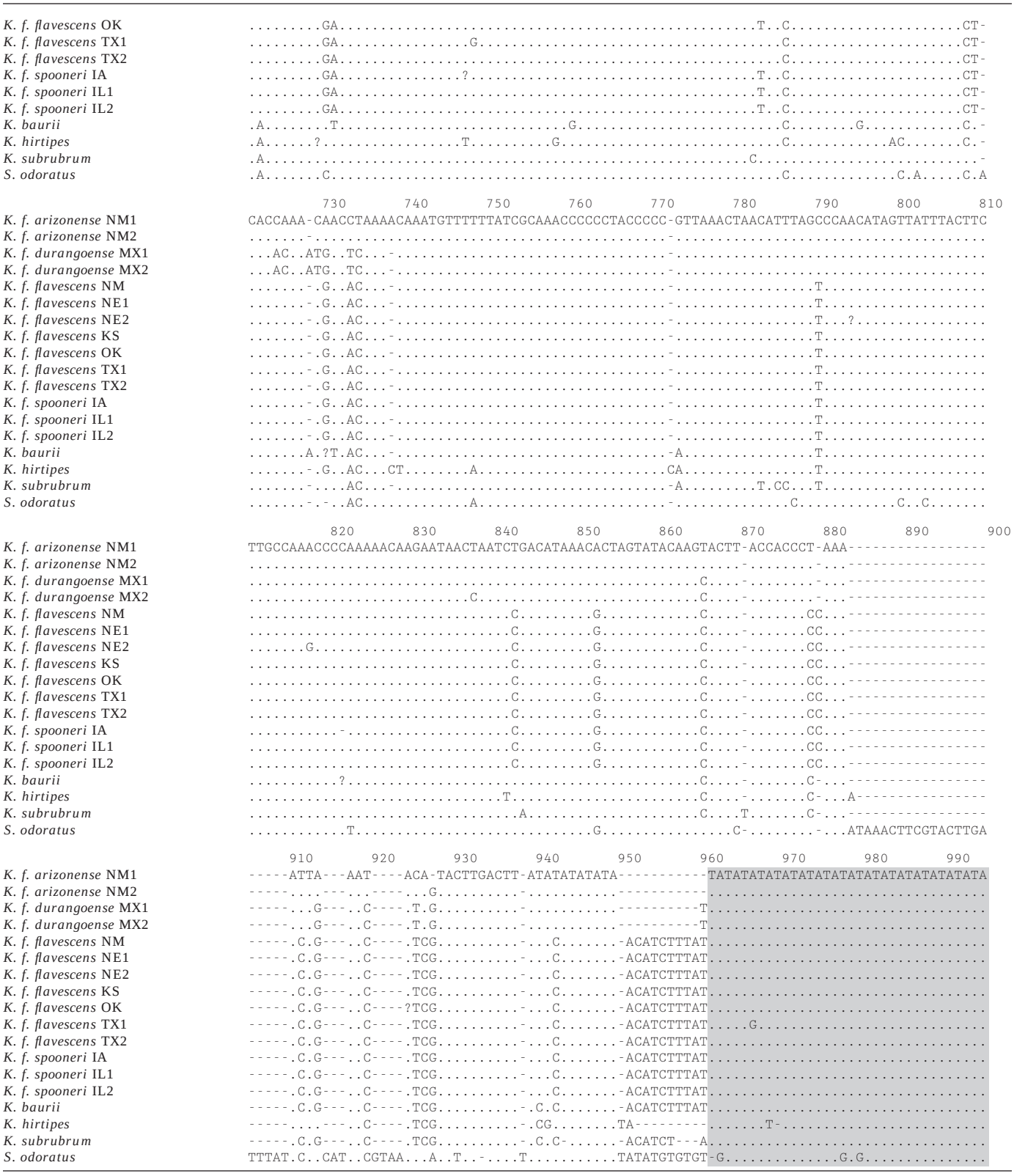
APPENDIX—*Continued*



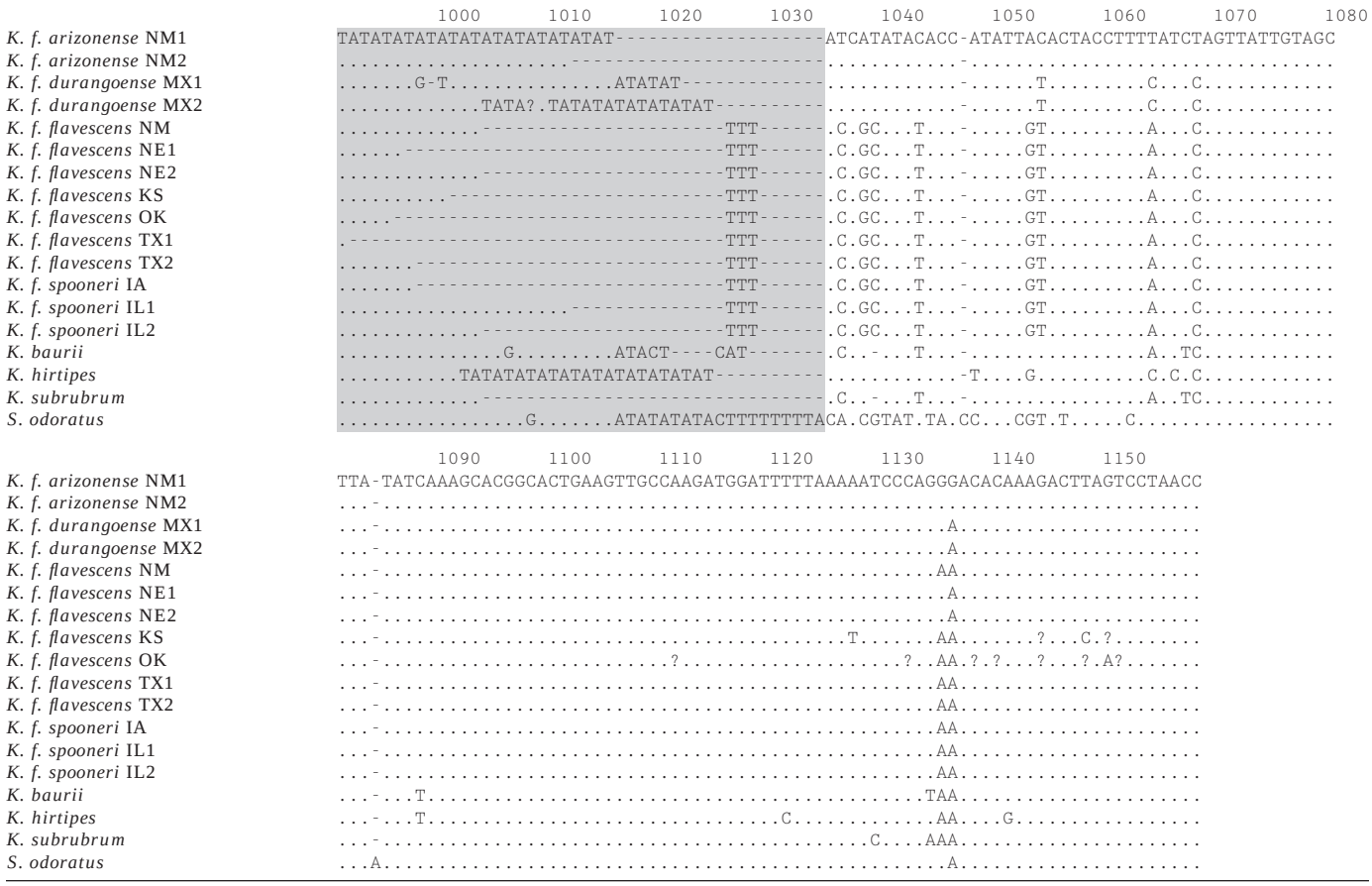
APPENDIX—*Continued*



APPENDIX—*Continued*



APPENDIX—*Continued*



Note. Dots represent the same nucleotide as the reference sequence (at top). Dashes designate gaps that are required for insertions and deletions in one or more members of the alignment. Shaded region (957–1034 bp) depicts the AT repeat region omitted from final analysis.

REFERENCES

Agassiz, L. (1857). "Contributions to the Natural History of the United States of America," Vols. 1 and 2, Little, Brown and Co., Boston.

Allard, M. C., Miyamoto, M. M., Bjornal, K. A., Bolten, A. B., and Bowen, B. W. (1994). Support for natal homing in green turtles from mitochondrial DNA sequences. *Copeia* **1994:** 34–41.

Aquadro, C. F., and Greenberg, B. D. (1983). Human mitochondrial DNA variation and evolution: Analysis of nucleotide sequences from seven individuals. *Genetics* **103:** 287–312.

Avise, J. C. (1986). Mitochondrial DNA and the evolutionary genetics of higher animals. *Phil. Trans. R. Soc. Lond. B* **312:** 325–342.

Avise, J. C., and Ball, R. M., Jr. (1990). Principles of genealogical concordance in species concepts and biological taxonomy. *In* "Oxford Surveys in Evolutionary Biology" (D. Futuyma and J. Antonovics, Eds.), Vol. 7, pp. 45–67. Oxford Univ. Press, London.

Avise, J. C., Bowen, B. W., Lamb, T., Meylan, A. B., and Bermingham, E. (1992). Mitochondrial DNA evolution at a turtle's pace: Evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Mol. Biol. Evol.* **9:** 457–473.

Baker, A. J., and Marshall, H. D. (1997). Mitochondrial control region sequences as tools for understanding evolution. *In* "Avian Molecular Evolution and Systematics" (D. P. Mindell, Ed.), pp. 51–82. Academic Press, San Diego.

Bennett, J. M. (1986). A method for sampling blood from hatchling loggerhead turtles. *Herpetol. Rev.* **17:** 43.

Bernatchez, L., Guyomard, R., and Bonhomme, F. (1992). DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Mol. Ecol.* **1:** 161–173.

Berry, J. F., and Berry, C. M. (1984). A re-analysis of geographic variation and systematics in the yellow mud turtle, *Kinosternon flavescens* (Agassiz). *Ann. Carnegie Mus. Nat. Hist.* **53:** 185–206.

Bickham, J. W., Lamb, T., Minx, P., and Patton, J. C. (1996). Molecular systematics of the genus *Clemmys* and the intergeneric relationships of emydid turtles. *Herpetologica* **52:** 89–97.

Bremer, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42:** 795–803.

Bremer, K. (1994). Branch support and tree stability. *Cladistics* **10:** 295–304.

Chang, D., and Clayton, D. A. (1985). Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. USA* **82:** 351–355.

Cohn, T. J. (1965). The arid land katydids of the North American genus *Neobarettia* (Orthoptera: Tettigonidae): Their systematics

and a reconstruction of their history. *Misc. Pub. Mus. Zool. Univ. Mich.* **126:** 1–180.

Cope, E. D. (1892). The Batrachia and Reptilia of northeastern Texas. *Proc. Acad. Nat. Sci. Philadelphia,* pp. 331–336.

Cracraft, J. (1982). Geographic differentiation, cladistics, and vicariance biogeography: Reconstructing the tempo and mode of evolution. *Am. Zool.* **22:** 411–424.

de Quieroz, K., and Donoghue, M. J. (1990). Phylogenetic systematics and species revisited. *Cladistics* **6:** 83–90.

Encalada, S. E., Lahanas, P. N., Bjorndal, K. A., Bolten, A. B., Miyamoto, M. M., and Bowen, B. W. (1996). Phylogeography and population structure of the Atlantic and Mediterranean green turtle *Chelonia mydas:* A mitochondrial DNA control region sequence assessment. *Mol. Ecol.* **1996:** 473–483.

Eriksson, T., and Wikstrom, N. (1995). "AutoDecay," Ver. 3.0 Botaniska Institutionen, Stockholm University, Stockholm.

Farris, J. S. (1974). Formal definitions of paraphyly and polyphyly. *Syst. Zool.* **23:** 548–554.

Felsenstein, J. (1993). "PHYLIP: Phylogenetic Inference Package," Ver. 3.5c, University of Washington, Seattle.

Gilmore, C. W. (1922). A new fossil turtle, *Kinosternon arizonense,* from Arizona. *Proc. U.S. Natl. Mus.* **62:** 1–8.

Good, S. V., Williams, D. F., Ralls, K., and Fleischer, R. C. (1997). Population structure of *Dipodomys ingens* (Heteromyidae): The role of spatial heterogeneity in maintaining genetic diversity. *Evolution* **51:** 1296–1310.

Hillis, D. M., and Huelsenbeck, J. P. (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *J. Hered.* **83:** 189–195.

Houseal, T. W., Bickham, J. W., and Springer, M. D. (1982). Geographic variation in the mud turtle, *Kinosternon flavescens. Copeia* **1982:** 567–580.

Iverson, J. B. (1979). A taxonomic reappraisal of the yellow mud turtle, *Kinosternon flavescens* (Testudines: Kinosternidae). *Copeia* **1979:** 212–225.

Iverson, J. B. (1981). Biosystematics of the *Kinosternon hirtipes* species group (Testudines: Kinosternidae). *Tul. Stud. Zool. Bot.* **23:** 1–74.

Iverson, J. B. (1989). The Arizona mud turtle, *Kinosternon flavescens arizonense* (Kinosternidae), in Arizona and Sonora. *Southwest. Nat.* **34:** 356–368.

Iverson, J. B. (1991a). Life history and demography of the yellow mud turtle, *Kinosternon flavescens. Herpetologica* **47:** 373–395.

Iverson, J. B. (1991b). Phylogenetic hypotheses for the evolution of modern Kinosternine turtles. *Herpetol. Monogr.* **5:** 1–27.

Iverson, J. B. (1998). Molecules, morphology, and mud turtle phylogenetics (Family Kinosternidae). *Chelonian Conserv. Biol.* **3:** 113–117.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16:** 111–120.

Lamb, T., Lydeard, C., and Gibbons, J. W. (1994). Molecular systematics of map turtles (*Graptemys*): A comparison of mitochondrial restriction site vs. sequence data. *Syst. Biol.* **43:** 543–559.

Lamb, T., and Osentoski, M. F. (1997). On the paraphyly of *Malaclemys:* A molecular genetic assessment. *J. Herpetol.* **31:** 258–265.

Maddison, W. P., and Maddison, D. R. (1992). "MacClade, Analysis of Phylogeny and Character Evolution," Ver. 3.07, Sinauer, Sunderland, MA.

Mahmoud, I. Y. (1969). Comparative ecology of the kinosternid turtles of Oklahoma. *Southwest. Nat.* **14:** 31–66.

Morafka, D. J. (1977). "A Biogeographical Analysis of the Chihuahuan Desert through Its Herpetofauna," Junk, The Hague.

Nei, M. (1987). "Molecular Evolutionary Genetics," Columbia Univ. Press, Columbia.

Osentoski, M. F., and Lamb, T. (1995). Intraspecific phylogeography of the gopher tortoise, *Gopherus polyphemus:* RFLP analysis of amplified mtDNA segments. *Mol. Ecol.* **1995:** 709–718.

Parker, A., and Kornfield, I. (1997). Evolution of the mitochondrial DNA control region in the *mbuna* (Cichlidae) species flock of Lake Malawi, East Africa. *J. Mol. Evol.* **45:** 70–83.

Parmley, D. (1992). Turtles from the late Hemphilian (latest Miocene) of Knox County, Nebraska. *Tex. J. Sci.* **44:** 339–348.

Riddle, B. R. (1995). Molecular biogeography in the pocket mice (*Perognathus* and *Chaetodipus*) and grasshopper mice (*Onychomys*): The Late Cenozoic development of a North American aridlands rodent guild. *J. Mammal.* **76:** 283–301.

Roman, J., Santhuff, S. D., Moler, P. E., and Bowen, B. W. (1999). Population structure and cryptic evolutionary units in the alligator snapping turtle. *Conserv. Biol.* **13:** 135–142.

Saccone, C., Pesole, G., and Sbisa, E. (1991). The main regulatory region of mammalian mitochondrial DNA: Structure–function model and evolutionary pattern. *J. Mol. Evol.* **33:** 83–91.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.

Seidel, M. E., Iverson, J. B., and Adkins, M. D. (1986). Biochemical comparisons and phylogenetic relationships in the family Kinosternidae (Testudines). *Copeia* **1986:** 285–294.

Smith, P. W. (1951). A new frog and a new turtle from the western Illinois sand prairies. *Bull. Chicago Acad. Sci.* **9:** 189–199.

Smith, P. W. (1957). An analysis of post-Wisconsin biogeography of the Prairie Peninsula region based on distributional phenomena among terrestrial vertebrate populations. *Ecology* **38:** 205–218.

Southern, S. O., Southern, P. J., and Dizon, A. E. (1988). Molecular characterization of a cloned dolphin mitochondrial genome. *J. Mol. Evol.* **28:** 32–42.

Starkey, D. E. (1997). "Molecular Systematics and Biogeography of the New World Turtle Genera *Trachemys* and *Kinosternon,*" Ph.D. dissertation, Texas A & M University, College Station, TX.

Swofford, D. L. (1998). "PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods)," Beta Ver. 4.0b2a, Sinauer, Sunderland, MA.

Templeton, A. R. (1983a). Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution* **37:** 221–244.

Templeton, A. R. (1983b). Convergent evolution and nonparametric inferences from restriction data and DNA sequences. *In* "Statistical Analysis of DNA Sequence Data" (B. S. Weir, Ed.), pp. 153–179. Dekker, New York.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.

Upholt, W. B., and Dawid, I. B. (1977). Mapping of mitochondrial DNA of individual sheep and goats: Rapid evolution in the D-loop region. *Cell* **11:** 571–583.

Walker, D., and Avise, J. C. (1998). Principles of phylogeography as illustrated by freshwater and terrestrial turtles in the southeastern United States. *Annu. Rev. Ecol. Syst.* **29:** 23–58.

Walker, D., Burke, V. J., Barák, I., and Avise, J. C. (1995). A comparison of mtDNA restriction sites vs. control region sequences in

phylogeographic assessment of the musk turtle (*Sternotherus minor*). *Mol. Ecol.* **4:** 365–373.

Walker, D., Nelson, W. S., Buhlmann, K. A., and Avise, J. C. (1997). Mitochondrial DNA phylogeography and subspecies issues in the monotypic freshwater turtle *Sternotherus odoratus*. *Copeia* **1997:** 16–21.

Walker, D., Ortì, G., and Avise, J. C. (1998). Phylogenetic distinctiveness of a threatened aquatic turtle (*Sternotherus depressus*). *Conserv. Biol.* **12:** 639–645.

Wright, H. E., Jr. (1968). History of the Prairie Peninsula. *In* "The Quaternary of Illinois" (R. E. Bergstrom, Ed.), pp. 78–88. Univ. of Illinois, College of Agriculture, Spec. Publ. 14.

Zink, R. M., and Blackwell, R. C. (1998a). Molecular systematics and biogeography of aridland gnatcatchers (Genus *Polioptila*) and evidence supporting species status of the California gnatcatcher (*Polioptila californica*). *Mol. Phylogenet. Evol.* **9:** 26–32.

Zink, R. M., and Blackwell, R. C. (1998b). Molecular systematics of the scaled quail complex (Genus *Callipepla*). *Auk* **115:** 349–403.