Molecular Phylogenetics and Evolution
Vol. 14, No. 3, March, pp. 414–422, 2000
doi:10.1006/mpev.1999.0718, available online at http://www.idealibrary.com on IDEAL®

Pre-Pleistocene Refugia and Differentiation between Populations of the Caucasian Salamander (*Mertensiella caucasica*)

David N. Tarkhnishvili,[1] Roger S. Thorpe, and J. W. Arntzen[2]

School of Biological Sciences, University of Wales, Brambell Building, Bangor LL57 2UW, United Kingdom

Received March 22, 1999; revised August 4, 1999

A 350-bp fragment of the mitochondrial cytochrome-*b* gene was sequenced in the Caucasian salamander, *Mertensiella caucasica*, representing 10 populations from across its range along the Black Sea coast. Five haplotypes were discovered among 65 fragments analyzed, differing at 2–50 positions. The highest differentiation between haplotypes was observed in animals from the eastern part of the species' range (Borjomi) compared to those from the remainder of the species' range. Randomly amplified nuclear DNA revealed a pattern of spatial genetic variation similar to that of the mitochondrial genome. *M. caucasica*, as currently known, represents two evolutionary lineages that evolved independently, perhaps since the lower Pliocene. These lineages represent taxa, possibly to be described as species, distributed in the Borjomi area in central Georgia and in southwestern Georgia and northeastern Turkey. The multivariate analysis of morphological data did not reveal significant differences between the taxa. However, substantial morphological differentiation was observed within both lineages, showing parallel patterns in body proportions and coloration patterns. This variation is possibly associated with extant ecological conditions. Salamanders with reduced pigmentation from southwestern Georgia were not genetically distinguishable from neighboring populations.

Key Words: **amphibians; Caucasus; cytochrome-*b* gene; historical biogeography; *Mertensiella caucasica;* morphological stasis; multivariate morphometrics; phylogeny; RAPD's.**

INTRODUCTION

The Caucasian salamander, *Mertensiella caucasica* (Waga, 1876), is one of at least six known representatives of the salamander subfamily Salamandrinae, which includes the genera *Chioglossa, Mertensiella,* and *Salamandra,* with a distribution over mountainous regions of Europe and the Near East (Nöllert and Nöllert, 1992; Griffiths, 1996). The sister taxon of *M. caucasica* is not the Anatolian *M. luschani* (Steindachner, 1891) but the Iberian *Chioglossa lusitanica* Bocage, 1864 (Titus and Larson, 1995; Veith *et al.,* 1998). The range of *M. caucasica* is restricted to the humid mountain forests of the northwestern Caucasus Minor in Turkey and Georgia (Fig. 1). The larger, western part of the range belongs to the Black Sea watershed, while the eastern part drains to the Caspian Sea. At a finer scale, *M. caucasica* has a fragmented distribution, with populations occurring along the upstream fragments of mountain brooks. Because of their specialized ecological requirements and patchy distribution, Tarkhnishvili and Serbinova (1993) and Tarkhnishvili (1994) postulated that local populations would be largely isolated and the species would be subjected to strong genetic subdivision. We employed a combination of genetic and multivariate morphometric techniques to test the prediction that *M. caucasica* is a highly differentiated taxon. The hypotheses tested are that the divergence between populations (1) is associated with geographical distance ("as the crow flies"), (2) is associated with geographical distance measured along river courses ("riverine distance"), and (3) reflects the geological history of the region. The latter hypothesis involves two separate refugias of subtropical humid forests on the eastern coast of the Black Sea associated with orogenic activity in the early Pliocene (Grossheim, 1948; Gvozdetsky, 1963).

MATERIAL AND METHODS

Muscular tissue was taken from tail tips from 73 individuals of *M. caucasica* from 10 populations spanning the entire species range. Populations were found in natural habitats within extensive areas of primary forest, with the exception of locality 8 (Batumi), which is in a mixed urban and agricultural setting. Altitudes ranged from 50 to 2000 m. For collecting details see Fig. 1 and Appendix 1.

[1] Present Address: Zoological Research Institute and Museum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany.

[2] Present address: Department of Zoology and Anthropology, University of Porto, CECA/ICETA/UP, Campus Agrário de Vairäo, 4480 Vila do Conde, Portugal.

1055-7903/00 $35.00

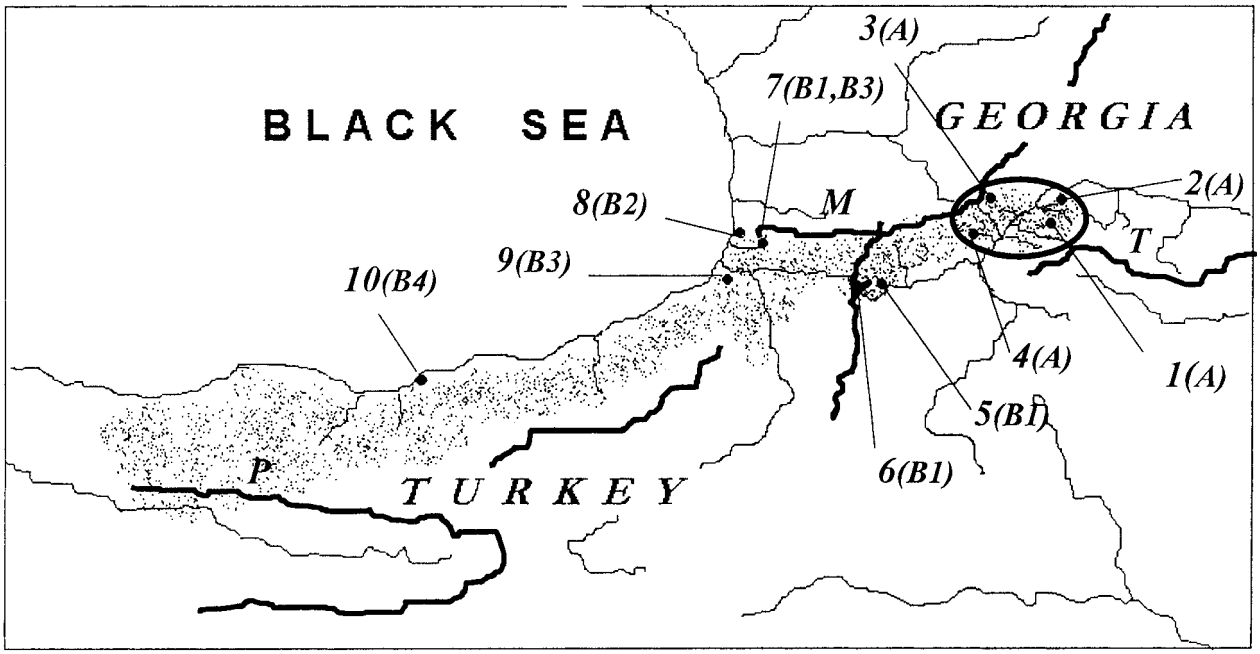

FIG. 1. The range of the Caucasian salamander, *Mertensiella caucasica* (modified from Tarkhnishvili, 1996), with study populations and localities numbered 1–10 (see Appendix 1). The shaded area indicate the distribution of the A-B4 mtDNA haplotypes. M, Meskheti mountains; P, Ponto mountains; and T, Trialeti mountains. The solid line limits the position of the hypothesized Pliocene refugium in the Borjomi area (Gvozdetsky, 1963).

DNA was extracted from alcohol-preserved tissue following the protocol of Sambrook *et al.* (1989). The primers MVZ15 (5′-GAACTAATGGCCCACAC(AA/TT)-TACGNAA-3′; Moritz *et al.,* 1992; Tan and Wake, 1995) and cyt*b*702 (5′-GGCAAATAGGAAGTATCATTCTG-3′; Moritz *et al.,* 1992, modified) were used for double-stranded amplification with *Taq* polymerase (Gibco BRL). The conditions were as follows: 33 cycles involving denaturation at 92°C for 90 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s. This yielded a ca. 800-bp-sized fragment of mitochondrial DNA representing a part of the cytochrome-*b* gene. The PCR product was purified with the wizard PCR Preps DNA Purification system. The dideoxy chain termination sequencing was performed using ^{35}S-labeled dATP (Sambrook *et al.,* 1989). We used negative controls in all PCR amplifications to assure that sequences were not from contaminated sources. Initially, 250-bp-long sequences were obtained for 45 samples. Sequences of 350 bp, corresponding to 16,279 (5′ end) and 16,629 (3′ end) of the *Xenopus laevis* mt DNA (Roe *et al.,* 1985) were obtained for 20 individuals (2 specimens per population).

Phylogenetic analysis of mtDNA haplotypes was carried out with PAUP 3.1.1 (Swofford, 1993) with the following parameter settings: (1) a uniform transition–transversion ratio (i.e., "no weighting"), (2) different weights assigned to transitions versus transversions inversely proportional to the frequency of different substitutions among ingroup taxa (all substitutions were used for calculating the ratio), and (3) transversions only. Published (García-París *et al.,* 1998) and unpublished (J. Alexandrino, pers. comm.) cytochrome-*b* sequences for *C. lusitanica, Salamandra atra,* and *S. salamandra* were employed as outgroups. Bootstrap replication scores (Felsenstein, 1985; Hillis and Bull, 1993) were determined over 10,000 heuristic search replicates to gain an impression of the strength of support from the data to the phylogenetic tree showing maximum parsimony. Evolutionary (patristic) distances between haplotypes were estimated with the Fitch–Margoliash (FM) algorithm to minimize the influence of uneven rates of mt-DNA change along lineages (Weir, 1996).

Randomly amplified DNA products (RAPD; Williams *et al.,* 1990) were studied in 58 individuals from nine localities (Appendix 1). The primer PR19 (5′-CTGGCG-GCTG-3′; Okamura *et al.,* 1993) was used for PCR amplification in a 50-μl volume for 40 cycles, involving denaturation at 94°C for 10 s, annealing at 42°C for 30 s, and extension at 73°C for 90 s. The PCR products were separated on agarose gels and stained with ethidiumbromide.

Matrices of genetic distance between populations were calculated from the mitochondrial data (FM distance measure between the modal cytochrome-*b* haplotypes) and from the nuclear DNA data (standardized Euclidian distance between RAPD profile, i.e., 1-band-sharing index). Three hypotheses on cause and effect of population substructuring in *M. caucasica* were formalized as follows: (1) geographic distance between popula-

TABLE 1

Variable Sites from the Aligned Sequences (350 bp of Mitochondrial Cytochrome-*b* Gene)

NP	14	*16*	*18*	19	26	32	33	44	47	56	59	65	68	78	80
A	C	*A*	*A*	A	T	C	C	A	G	G	C	A	C	C	A
B1	C	*G*	*A*	A	C	C	T	C	A	C	C	G	T	T	G
B2	C	*G*	*A*	A	C	C	T	C	A	C	C	G	T	T	G
B3	C	*G*	*A*	A	C	C	C	C	A	C	C	G	T	T	G
B4	T	*A*	*G*	G	C	T	T	T	A	C	T	G	C	T	G
	87	89	110	116	123	128	134	137	140	146	*151*	155	173	176	188
A	*G*	T	C	C	C	C	C	C	G	C	*G*	C	T	C	C
B1	*A*	T	T	T	T	T	T	T	G	T	*A*	C	C	T	T
B2	*A*	T	C	T	T	T	T	T	G	T	*A*	C	C	T	T
B3	*A*	T	T	T	T	T	T	T	G	T	*A*	C	C	T	T
B4	*A*	C	T	T	C	T	T	T	A	T	*A*	A	C	T	C
	197	200	215	218	224	227	*236*	244	*246*	247	*251*	256	272	275	*276*
A	C	C	T	T	G	C	*T*	C	*T*	C	*T*	C	C	A	*T*
B1	T	T	T	C	A	T	*T*	T	*T*	T	*A*	T	A	C	*T*
B2	T	T	T	C	A	T	*T*	T	*T*	T	*A*	T	A	C	*T*
B3	T	T	T	C	A	T	*T*	T	*T*	T	*A*	T	A	C	*T*
B4	T	T	C	C	A	T	*C*	C	*C*	T	*A*	C	C	C	*C*
	278	*279*	281	287	290	296	299	320	*322*	323	326	335	339	342	348
A	C	*T*	C	A	T	A	T	T	*T*	T	T	C	G	T	T
B1	T	*T*	T	A	C	G	C	C	*T*	G	C	T	G	C	C
B2	T	*T*	T	A	C	G	T	C	*T*	G	T	T	G	C	C
B3	T	*T*	T	G	C	G	C	C	*T*	G	C	T	G	C	C
B4	T	*C*	T	A	C	A	C	C	*C*	G	C	T	A	C	C

Note. A–B4, haplotypes (see text); NP, position of a variable bp in the aligned sequence. Variable sites corresponding to variations in the encoded aminoacids are shown in italics.

tions was calculated from geographical coordinates, (2) riverine distance was measured on a digitizing tablet from maps at scale 1/200,000 (Ministry of Defence of the USSR, 1978), and (3) the position of each population was determined as within or outside the borders of the hypothesized Pliocene refugium in the upper currents of the Kura river (Djavakhishvili *et al.,* 1964; Fig. 1). To test for the statistical significance of alternative hypotheses explaining observed genetic distances, partial Mantel permutation tests were applied on standardized distance matrices with 10,000 randomizations (Manly, 1986; Thorpe *et al.,* 1995).

Fifteen continuous, one meristic, and seven qualitative descriptors of size and coloration pattern were taken for 68 adult salamanders from four populations (see Appendix 1 for sample size). Characters are the following: SVL, snout–vent length, from the tip of the snout to the posterior edge of the cloacal cleft; TL, tail length, such that SVL plus TL is total length; HL, head length, from the tip of the snout to throat fold; HW, head width at the widest point; IOD, interorbital distance; IND, distance between nostrils; NED, distance from nostril to eye; PL, parotoid length; FLL, forelimb length; 3FL, length of the third finger; HLL, hindlimb length; 4TL, length of the fourth toe; ILD, interlimb distance; CCL, length of the cloacal cleft; SL, length of the spike on the base of the tail (males only); and CGC, number of costal grooves. The following coloration characters were scored binary (yes/no): YS, dorsal yellow spots arranged in two tracks (versus scattered or absent); SO, dorsal spots oblong shape (versus round or irregular); SL, dorsal spots large (versus small or almost absent); SE, dorsal spots sharp edged (versus border ill defined); SN, number of dorsal spots on the left lateral side <10 (versus $=$ or >10); DC, dorsal coloration reddish-brown (versus dark-brown or black); VS, ventral side densely covered with silver-white speckles (versus speckles few or absent); and DP, small dorsal protuberances present (versus absent). Morphological data were not available for populations in which sampling was based on juveniles and larvae (see Appendix 1).

Measurements were taken with precision (0.1 mm) callipers and ln-transformed. The standardized residuals of the regression of each character on snout–vent length were calculated, thereby reducing the number of morphometric variables to 14. This transformation was done to reduce the effect of nonlinear correlation between separate proportions and individual size (Thorpe and Leamy, 1983; Sokal and Rolf, 1995). Principal component analysis (PCA) was applied to the continuous and discrete variables separately. Two-way ANOVA detected significant differences in 4 morphometric characters for males and females ($P < 0.01$, results not shown) and therefore the results are presented for males and females separately. Statistical analysis was carried out with SPSS 6.1 (SPSS, 1994).

RESULTS

Analysis of Mitochondrial DNA Sequences

Sixty variable positions (17%) were recorded in the 350-bp cytochrome-*b* fragment shaping five different *M. caucasica* haplotypes (Table 1; GenBank Accession Nos. AF170013–AF170016). These haplotypes differed from the outgroup haplotype sequences in 18–21% (63–75 positions). Haplotype *A* differed from the other *Mertensiella* haplotypes in 12–14% while haplotypes *B1–B4* differed among themselves by 0.6–6.3% (Table 2). The most parsimonious hypothesis on haplotype evolution is presented in Fig. 2. Tree topologies calculated under different weighting regimes are not contradictory. The five *Mertensiella* haplotypes are monophyletic with respect to the outgroup with a bootstrap support of 100%. The haplotypes *B1–B4* form a monophyletic group with a bootstrap support of 98–100% and *B1–B3* form a monophyletic group with a bootstrap support of 86%. Among 116 amino acids coded by the sequenced fragment, variation was observed at 10 positions (Table 1).

Haplotype *A* was found at localities 1–4, *B1* was found at localities 5 and 6, *B2* was found at locality 8, *B3* was found at locality 9, and *B4* was found at locality 10. At locality 7 two haplotypes (*B1* and *B3*) were observed in a 1:4 ratio.

Randomly Amplified DNA Products

The PCR products obtained by single-stranded amplification with primer P19 resolved 16 bands in the size range of 300–600 bp. Polymorphisms were observed involving five characters and four of these distinguished individuals possessing haplotype *A* from individuals possessing haplotypes *B* (Fig. 3). A single polymorphism was found in individuals with haplotype *A*. No differences in RAPD banding pattern were found between individuals with a *B* haplotype.

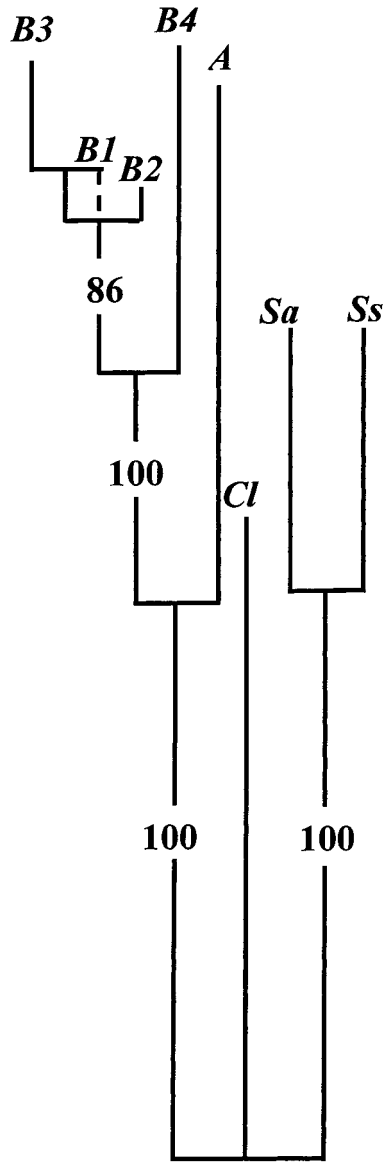

FIG. 2. Phylogenetic relationships between cytochrome-*b* haplotypes of *Mertensiella caucasica* (A–B4), with outgroup species *Chioglossa lusitanica, Salamandra atra,* and *S. salamandra,* inferred by maximum-parsimony analysis. Percentage bootstrap support is indicated along branches (branch length reflects patristic FM distances between haplotypes).

TABLE 2

The Absolute and Relative Substitution Frequencies (%) Observed between Haplotypes of *Mertensiella caucasica* and Various Outgroups[a]

	Ss	*Sa*	*Cl*	*A*	*B1*	*B2*	*B3*	*B4*
Ss		6.6	17.8	18.9	19.1	19.1	18.9	21.4
Sa	23		19.3	19.4	21.1	21.1	20.6	21.1
Cl	63	64		19.7	18.9	18.9	18.9	18.0
A	66	68	69		12.6	11.7	12.6	14.3
B1	67	74	66	44		0.9	0.6	5.4
B2	67	74	66	41	3		1.4	6.3
B3	66	72	66	44	2	5		6.0
B4	75	74	63	50	19	22	21	

[a] Ss, *Salamandra salamandra;* Sa, *S. atra* (García-París *et al.,* 1998); Cl, *Chioglossa lusitanica* (J. Alexandrino, personal communication); *A–B4* are the haplotypes observed in *M. caucasica.*

Matrix Correspondence Tests

Haplotype genetic distance between populations (Table 3) was significantly associated with the hypothesized Pliocene refugium ($P < 0.01$) and not with geographic or riverine distance (Table 3) ($P > 0.05$). Similarly, a significant association was observed between RAPD-derived genetic distance and the hypothesized refugia ($P < 0.01$) and not with geographic or riverine distance ($P > 0.05$). A significant association was also observed between genetic distance matrices con-

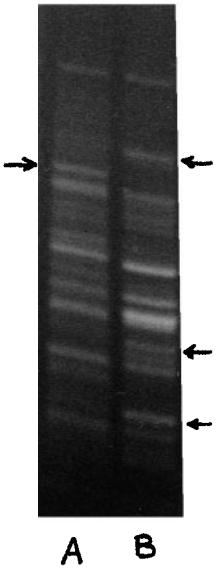

FIG. 3. RAPD profile of two specimens of *Mertensiella caucasica* representing lineages with haplotypes A and B1–4. Characteristic bands are indicated by arrows.

structed for mtDNA haplotypes and the RAPD profiles ($P < 0.05$).

Multivariate Morphological Analysis

The highest loadings on the first PCA axis for body proportions, explaining 45% of the total observed variance, were recorded for the characters tail length, head width, nostril–eye distance, interlimb distance, and cloacal cleft length (Table 4). The second axis, explaining 13% of the observed variance, was dominated by a single character, the length of the third finger. In males, these axes helped to distinguish populations 3 and 6 from populations 1 and 7 (Fig. 4).

In the analysis of coloration pattern, the first and second PCA axis explained 32 and 23% of the observed variance, respectively. High loadings were recorded for the characters "spots edge" (SE) and "dorsal coloration" (DC) along the first axis and for the characters "spot size" (SL) and "spot number" (SN) along the second axis (Table 4). In combination, these axes separated individuals from localities 1 and 7, while the individuals from the localities 3 and 6 kept an intermediate position.

DISCUSSION

Two geographically distinct evolutionary lineages are found within the range of *M. caucasica,* as evidenced by the analysis of cytoplasmic and nuclear genes. The level of differentiation between them exceeds those between some fairly distinct urodele species, such as *Euproctus montanus* and *E. platycephalus, Taricha torosa* and *T. granulosa,* and *Salamandra salamandra* and *S. atra* (Caccone *et al.,* 1997; Tan and Wake, 1995; García-París *et al.,* 1998). This observation raises two questions: (1) how old are these lineages and

TABLE 3

Geographic Distance, Riverine Distance, and Genetic Distance Observed between *Mertensiella caucasica* Populations

Population	1	2	3	4	5	6	7	8	9	10
1	0	4	12	23	84	89	148	153	163	275
2	6	0	16	27	88	93	152	157	167	280
3	27	24	0	13	74	79	136	143	154	260
4	48	46	14	0	60	65	123	128	140	250
5	117	114	103	80	0	5	64	71	80	185
6	122	120	109	86	6	0	59	66	75	180
7	218	216	205	182	96	90	0	12	16	135
8	232	230	219	196	110	104	17	0	16	125
9	226	224	213	190	104	98	23	43	0	115
10	366	364	353	330	244	238	163	183	140	0
1		0	0	0	0.126	0.126	0.124	0.141	0.124	0.144
2	0		0	0	0.126	0.126	0.124	0.141	0.124	0.144
3	0.24	0.24		0	0.126	0.126	0.124	0.141	0.124	0.144
4	0	0	0.24		0.126	0.126	0.124	0.141	0.124	0.144
5	1	1	0.85	1		0	0.012	0.015	0.012	0.068
6	1	1	0.85	1	0		0.012	0.015	0.012	0.068
7	1	1	0.85	1	0	0		0.012	0	0.067
8	1	1	0.85	1	0	0	0		0.014	0.083
9	1	1	0.85	1	0	0	0	0		0.067

Note. Upper panel to the right of the diagonal—geographical "as the crow flies" distance (km); below the diagonal—riverine distances (km, details see text). Lower panel to the right of the diagonal—FM patristic distances between modal haplotypes; below the diagonal—Euclidean distances between RAPD profiles, based on frequencies of six polymorphic bands with length 300–600 bp.

TABLE 4

Percentage Variance Explained and Scores of the First Two Axes of a Principal Component Analysis for Body Proportions and Coloration Patterns Observed in *Mertensiella caucasica*

	First	Second
	Body proportions	
% Variance	44.6	12.8
TL	0.85	0.25
HL	0.46	0.57
HW	0.76	0.04
IOD	0.69	0.21
IND	0.68	−0.46
NED	0.74	0.16
PL	0.56	−0.46
FLL	0.70	0.01
3FL	0.04	0.82
HLL	0.60	0.02
4TL	0.68	−0.47
ILD	0.75	−0.07
CCL	0.83	0.14
CGC	0.61	0.02
	Coloration patterns	
% Variance	31.6	23.4
SO	0.42	−0.48
SL	0.18	0.87
SE	0.88	0.17
YS	0.53	−0.38
SN	0.13	0.81
DC	−0.83	0.12
VS	−0.65	−0.20
DP	0.35	−0.05

(2) do they represent separate species? An associated question is why the deep phylogenetic differentiation of *M. caucasica* is not reflected by morphological change.

The "molecular clock" for substitutions in the cytochrome-*b* gene of salamanders has been calibrated at 7–8% per 10 mA (Spolsky *et al.,* 1992; Tan and Wake, 1995) and at 0.9% per 10 mA if only transversions are taken into consideration (Caccone *et al.,* 1997). Under the assumption that base substitutions follow a Poisson process, the probability of obtaining the observed divergence value (12%) for the analyzed mt-DNA fragments over a time span of 2 mA (i.e., during the Pleistocene) is small, even when broad confidence limits for molecular clock estimates are taken into consideration ($P < 0.001$; see Hillis *et al.,* 1996). We conclude that the separate evolution of two lineages originated well before the Pleistocene.

During the Miocene, the Great Caucasus formed an island in the central part of the Paratethys Sea (Ruggieri, 1967; Kholodov and Nedumov, 1996) that became connected to Asia Minor in the upper Miocene (Vereshchagin, 1959; Tuniyev, 1990). During the early Pliocene, orogenic activity resulted in the formation of extensive mountains in this region. This process supposedly restricted the subtropical forests of the Colchis (the western part of the Caucasus Isthmus) into several refugias (Grossheim, 1948; Vardanyants, 1948; Maruashvili, 1952; Gvozdetsky, 1963). Currently, one center of distribution of *M. caucasica* covers the eastern part of the Black Sea basin.

A smaller center of distribution exists in the Borjomi area in the mountains bordering the Kura river (Djavakhishvili *et al.,* 1964). The localities of the Caucasian salamander attributable to the eastern lineage are from this refugium (Fig. 1). The split between the lineages may have started before the conjunction of the Caucasus island and Asia Minor. The absence of *Mertensiella* from the Great Caucasus is in line with this scenario, because, orogenically, the Minor Caucasus is associated with the Anatolian plateau and not with the Great Caucasus (Beruchashvili *et al.,* 1986). The relatively large genetic distance between some of the *B* haplotypes may reflect the fragmentation of subtropical forests in the Pliocene.

Allocation of species status to eastern and western Caucasian *Mertensiella* depends to a large extent on what species concept one would wish to apply. Reproductive isolation of lineages would indicate that they are different biological species (Mayr, 1969), while the evolutionary independence of lineages would indicate that they are different evolutionary species (Wiley, 1978; Frost and Hillis, 1990). With the current state of knowledge, a decision about reproductive isolation between the eastern taxon and the western taxon cannot be made, precluding the application of the biological species concept. The particular states of some nuclear and cytoplasmic genetic characters define the lineages, indicate evolutionary independence, and support species status according to the evolutionary species concept.

According to Veith *et al.* (1998), *M. caucasica* shares ancestry with *C. lusitanica* at 14–15 mA. The further genetic differentiation of *M. caucasica* appears to have been accompanied by morphological stasis. The reproductive patterns, breeding behavior, and embryonic development in the two lineages of *M. caucasica* appear to be similar (Tarkhnishvili and Serbinova, 1993, 1997; Schultschik, 1994a,b). Tartarashvili and Bakradze (1989) described salamanders from locality 7 as the subspecies *djanashvilii.* Although we observed some morphological variation between populations, the differences appear to reflect not phylogeny but ecological conditions. Genetically distant individuals from localities 3 and 6 were more similar to each other than to genetically related individuals from populations 1 and 7. This was the case both for body proportions and coloration patterns. An analogous situation was described for the tailed amphibian *Taricha torosa,* in which genetically unrelated mountain populations display similar coloration patterns (Tan and Wake, 1995).

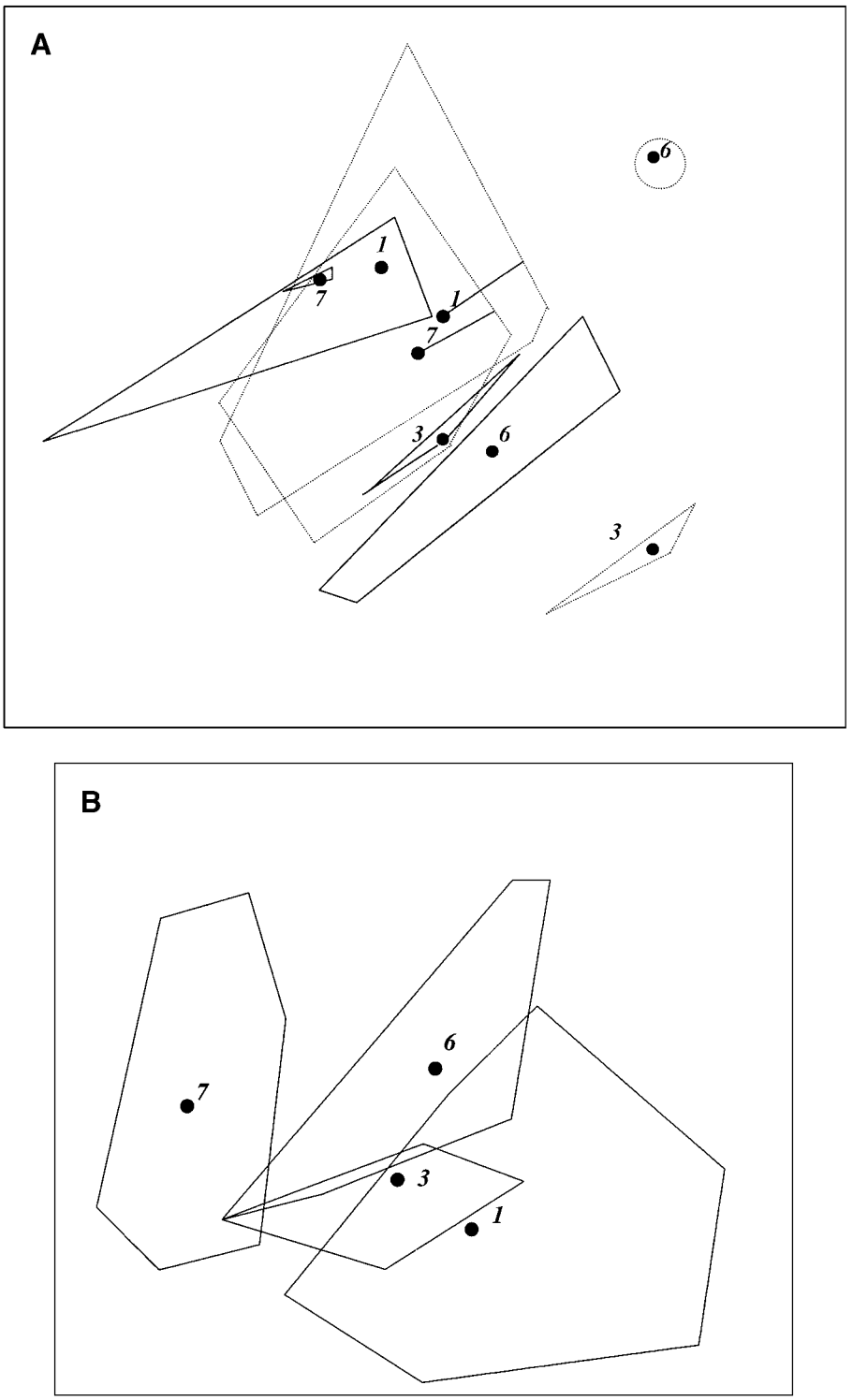

FIG. 4. Multivariate analysis of body proportions and coloration patterns as observed in *Mertensiella caucasica.* Bivariate plots for males with solid dots and females with open dots: (a) principal component analysis for body proportions and (b) principal component analysis for coloration patterns with sexes pooled.

APPENDIX 1

Geographical and Ecological Description of Sampling Localities

Locality 1—Kekia, at 41° 52′N, 43° 30′E ($N = 36$; $N1 = 8$; $N2 = 2$; $N3 = 10$; $N4 = 26$). Mountain brook; second-order tributary of the river Kura. Mixed forest at 1300–1400 m altitude; annual precipitation 1000 mm; snow cover 4–5 months per year.

Locality 2—Kamis Vake, at 41° 55′N, 43° 33′E ($N = 4$; $N1 = 1$; $N2 = 2$; $N3 = 3$). Further as number 1.

Locality 3—Baniskhevi, at 41° 52′N, 43° 16′E ($N = 12$; $N1 = 10$; $N2 = 2$; $N3 = 9$; $N4 = 7$). First-order tributary stream of the river Kura. Humid deciduous forest at 1400–1500 m; annual precipitation 1300 mm; snow cover 4–5 months.

Locality 4—Chitakhevi, at 41° 47′N, 43° 15′E ($N = 5$; $N1 = 3$; $N2 = 2$; $N3 = 5$). First-order tributary stream of the river Kura. Hornbeam forest at 1200–1400 m; annual precipitation 1000 mm; snow cover 4–5 months.

Locality 5—Goderdzi Pass site A, at 41° 38′N, 42° 35′E ($N = 2$; $N1 = 0$; $N2 = 2$; $N3 = 2$). Third-order tributary stream of the river Kura. Subalpine pine and spruce forest at 1700 m; annual precipitation 1000 mm; snow cover 5–6 months.

Locality 6—Goderdzi Pass site B, at 41° 36′N, 42° 28′E ($N = 9$; $N1 = 7$; $N2 = 2$; $N3 = 7$; $N4 = 9$). Third-order tributary stream of the river Kura. Subalpine meadows with stands of pine at 2000 m; annual precipitation 1700 mm; snow cover at least 6 months.

Locality 7—Mtirala Mts., at 41° 36′N, 41° 50′E ($N = 26$; $N1 = 8$; $N2 = 2$; $N3 = 10$; $N4 = 26$). First-order tributary of the river Korolistskali. Beech forest with evergreen undergrowth at 1300–1400 m; annual precipitation over 4000 mm; snow cover 3–4 months.

Locality 8—Batumi botanical garden, at 41° 43′N, 41° 43′E ($N = 4$; $N1 = 2$; $N2 = 2$; $N3 = 4$). Small brook discharging directly into the Black Sea. Small plot of semi-natural Colchic-type forest surrounded by oranges and tea plantations at 30–50 m; annual precipitation 2200 mm; no lasting snow cover.

Locality 9—Charnali, 41° 33′N, 41° 36′E ($N = 8$; $N1 = 6$; $N2 = 2$; $N3 = 8$). Brook; second-order tributary of the river Chorokh. Deciduous forest with evergreen undergrowth at 400 m; annual precipitation 2100 mm; no lasting snow cover.

Locality 10—Erikli, Turkey, 40° 54′N, 39° 30′E ($N = 2$; $N2 = 2$). Tributary of the river Kale. 1000 m; annual precipitation at Trabzon (50 m) 830 mm; 8 frost days per year.

Note. Sample sizes are coded as follows: N, total sample size; $N1$, 250-bp mtDNA fragment; $N2$, 350-bp mtDNA fragment; $N3$, RAPD profile; $N4$, adult morphology. Vouchers are placed in the collection of Tbilisi State University. Data sources: Anonymous (1996), Ministry of Defence of the USSR (1978), and Djavakhishvili *et al.* (1964).

ACKNOWLEDGMENTS

We thank R. Gokhelashvili for assistance in collecting samples in Georgia; K. Olgun, S. Tunçer, and A. Zuiderwijk for the sample from Turkey; and J. Alexandrino for unpublished sequence data. O. Gaggiotti, M. García-París, T. Griffiths, N. Hardman, A. Hille, and anonymous referees provided valuable suggestions to an earlier version of the manuscript. D.N.T. was supported by a NATO/Royal Society post-doctoral fellowship.

REFERENCES

Anonymous. (1996). "Statistical Yearbook of Turkey," State Institute of Statistics, Prime Ministry Republic of Turkey, Ankara.

Beruchashvili, N. L., Gabrielyan, G. K., and Gajiev, F. A. (1986). "Physical Geography of Transcaucasia." Erevan State Univ. Publication, Erevan, Armenia. [in Russian]

Caccone, A., Milinkovitch, M. C., Sbordoni, V., and Powell, J. R. (1997). Mitochondrial DNA rates and biogeography in European newts (genus *Euproctus*). *Syst. Biol.* **46:** 126–144.

Djavakhishvili, A. N., Aslanikashvili, A. F., and Dzotsenidze, G. S. (1964). "Atlas of the Georgian Soviet Socialist Republic." Head Department of Geodezy and Cartography of the USSR, Tbilisi, Moscow. [in Russian].

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

Frost, D. R., and Hillis, D. M. (1990). Species in concept and practice: Herpetological applications. *Herpetologica* **46:** 87–103.

García-París, M., Alcobendas, M., and Alberch, P. (1998). DNA evolution of *Salamandra salamandra* (Caudata: Salamandridae) from Southern Spain. *Copeia* **1998:** 173–176.

Griffiths, R. A. (1996). "Newts and Salamanders of Europe," Poyser, London.

Grossheim, A. A. (1948). "Vegetation Cover of the Caucasus," MOIP, Moscow. [in Russian].

Gvozdetsky, N. A. (1963). "Caucasus," Geografgiz, Moscow. [in Russian].

Hillis, D. M., Moritz, C., and Mable, B. C. (1996). "Molecular Systematics," Sinauer, Sunderland, MA.

Hillis, D. M., and Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* **42:** 182–192.

Kholodov, V. N., and Nedumov, R. I. (1996). Problems of the Caucasus Palaeoland existence during the Oligocene–Miocene time. *Stratigr. Geol. Correlation* **4:** 181–190.

Manly, B. F. J. (1986). Randomization and regression methods for testing associations with geographical, environmental and biological distances between populations. *Res. Pop. Ecol.* **28:** 201–218.

Maruashvili, L. I. (1952). "Main Problems of Late-Kaenozoan History of Landscapes of the Caucasus Isthmus," Geogr. Society of USSR Publ., Moscow. [in Russian]

Mayr, E. (1969). "Principles of Systematic Zoology," McGraw-Hill, New York.

Ministry of Defense of the USSR. (1978). Topographic maps, scale 1:200,000.

Moritz, C., Schneider, C. J., and Wake, D. B. (1992). Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Syst. Biol.* **41:** 273–291.

Nöllert, A., and Nöllert, C. (1992). "Die Amfibien Europas," Franck-Kosmos Verlags, Stuttgart.

Okamura, B., Jones, C. S., and Noble, L. R. (1993). Randomly amplified polymorphic DNA analysis of clonal population structure and geographic variation in a freshwater bryozoan. *Proc. R. Soc. Lond. B* **253:** 147–154.

Roe, B. A., Ma, D.-P., Wilson, R. K., and Wong, J. F.-H. (1985). The complete nucleotide sequence of the *Xenopus laevis* mitrochondrial DNA genome. *J. Biol. Chem.* **260:** 9759–9774.

Ruggieri, G. (1967). The Miocene and later evolution of the Mediterranean Sea. *In* "Aspects of Tethyan Biogeography" (C. G. Adams and D. V. Ager, Eds.), pp. 283–290. Syst. Assoc. Publ., London.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schultschik, G. (1994a). Zur Fortpflanzungsbiologie von Mertensiella caucasica (Waga, 1976) (Urodela: Salamandridae). *Abhandl. Berichte Naturkunde Magdeburg* **17:** 163–175.

Schultschik, G. (1994b). Mertensiella caucasica: Haltung, Nachzucht und Freiland-beobachtungen. *Salamandra* **30:** 161–173.

Sokal, R. R., and Rohlf, F. J. (1995). "Biometry," 3rd ed., Freeman, New York.

Spolsky, C. M., Philips, C. A., and Uzzell, T. (1992). Antiquity of clonal salamander lineages revealed by mitochondrial DNA. *Nature* **365:** 706–708.

SPSS (1994). SPSS for windows, release 6.1, SPSS, Chicago.

Swofford, D. L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, version 3.1, Computer program, Illinois Natural History Survey, Champaign, IL.

Tan, A. M., and Wake, D. B. (1995). MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata, Salamandridae). *Mol. Phylogenet. Evol.* **4:** 383–394.

Tarkhnishvili, D. N. (1994). Interdependences between populational, developmental and morphological features of the Caucasian salamander, *Mertensiella caucasica. Mertensiella* **4:** 312–325.

Tarkhnishvili, D. N. (1996). The distribution and ecology of the amphibians of Georgia and the Caucasus: A biogeographical analysis. *Zeitschr. Feldherpetol.* **3:** 167–196.

Tarkhnishvili, D. N., and Serbinova, I. A. (1993). The ecology of the Caucasian salamander in a local population. *Asiatic Herpetol. Res.* **5:** 147–165.

Tarkhnishvili, D. N., and Serbinova, I. A. (1997). Normal development of the Caucasian salamander (*Mertensiella caucasica*). *Adv. Amphibian Res. Former Soviet Union* **2:** 13–30.

Tartarashvili, R. V., and Bakradze, M. A. (1989). A new subspecies of the Caucasian salamander. *Bull. Acad. Sci. Georgia* **133:** 177–179. [in Russian]

Thorpe, R. S., and Leamy, L. (1983). Morphometric studies in inbred and hybrid house mice (*Mus* sp.): Multivariate analysis of size and shape. *J. Zool.* **199:** 421–432.

Thorpe, R. S., Malothra, A., Black, J. C., Daltry, J. C., and Wuster, W. (1995). Relating geographic pattern to phylogenetic process. *Phil. Trans. R. Soc. London B* **349:** 61–68.

Titus, T. A., and Larson, A. (1995). A molecular phylogenetic perspective on the evolutionary radiation of the salamander family Salamandridae. *Syst. Biol.* **44:** 125–151.

Tuniyev, B. S. (1990). On the independence of the Colchic center of amphibian and reptile speciation. *Asiatic Herpetol. Res.* **3:** 67–84.

Vardanyants, L. A. (1948). "Late-Pliocene History of the Caucasian–Black Sea–Caspian Region," Armen. SSR Acad. Sci. Publ., Erevan.

Veith, M., Steinfartz, S., Zardoya, R., Seitz, A., and Meyer, A. (1998). A molecular phylogeny of 'true' salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes. *J. Zool. Syst. Evol. Res.* **36:** 7–16.

Vereshchagin, N. K. (1959). "Mammals of Caucasus: Genesis of the Terrain Fauna," USSR Acad. Sci. Publ., Moscow-Leningrad. [in Russian]

Weir, B. S. (1996). "Genetic Data Analysis II," Sinauer, Sunderland, MA.

Wiley, E. O. (1978). The evolutionary species concept reconsidered. *Syst. Zool.* **27:** 17–26.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.* **18:** 1631–1635.