

## Effects of Holocene Climate Fluctuation on Mitochondrial DNA Variation in the Ringed Salamander, *Ambystoma annulatum*

CHRISTOPHER A. PHILLIPS, GLORIA SUAU, AND ALAN R. TEMPLETON

We analyzed mitochondrial DNA variation in the Ringed Salamander, *Ambystoma annulatum*. Four of six populations studied were fixed for the same haplotype. We attribute this lack of detectable variation to recent colonization of this area, a hypothesis which is supported by paleoecological data and previous biogeographical analysis of the sympatric Spotted Salamander, *Ambystoma maculatum*. This study demonstrates the potential benefits of comparing biogeographical data across species and suggests that the Hypsithermal Interval may be an overlooked source of important historical impacts for species in the midwestern United States.

GEOGRAPHIC structure of genetic variation is influenced by recurrent events such as restricted gene flow and nonrecurrent, historical events such as range expansion and long-distance colonization (Templeton et al., 1995). The relative importance of these factors to population structure varies among populations and species, but it is becoming clear that historical factors can play a major role (Brawn et al., 1996), especially in species with limited vagility (Larson et al., 1984; Phillips, 1994). A species' geographic range can also determine whether recurrent or historical factors are more influential; for example, historical factors should predominate in organisms that currently inhabit recently glaciated portions of North America and Europe (Sage and Wolff, 1986; Merila et al., 1996; Bernatchez and Wilson, 1998). Other, less well-known climatic events may be equally important determining factors and can involve species whose current ranges were not directly impacted by the presence of ice sheets.

The Hypsithermal Interval, a warm-dry period beginning about 8000 years ago and lasting until 4000 years before present (Wright, 1971; Webb and Bryson, 1972) is thought to have caused the expansion of the Prairie Peninsula beyond its current boundaries in midwestern North America. Across large sections of Illinois and Missouri, deciduous forest was replaced by xeric-adapted grassland communities such as prairie and savanna. One implication of this vegetation shift is that woodland species currently inhabiting the borders of the Prairie Peninsula must have colonized these regions in the last 4000 years when deciduous forest was rees-

tablished as the climate ameliorated. Phillips (1994) postulated that this recent invasion, coupled with waves of colonization and extinction, explained the lack of mtDNA variation detected in Spotted Salamanders, *Ambystoma maculatum*, along the current southern boundary of the Prairie Peninsula in the northern Missouri Ozarks. This study provides a test of this hypothesis by examining mtDNA variation in another woodland salamander from this region, the Ringed Salamander, *A. annulatum*. This species was chosen because it is restricted to hardwood or mixed hardwood-pine forests and the northern extent of its current range coincides with the southeastern boundary of the Prairie Peninsula.

### MATERIALS AND METHODS

Ringed salamanders are found throughout the forests of the Ozark Plateau from St. Louis, Missouri, southwest to northeastern Oklahoma and northwestern Arkansas (Fig. 1). We sampled 101 ringed salamanders from six populations throughout the species' range in Missouri (Table 1). Populations are defined as the individuals breeding in a single pond. Four populations were sampled in the northern Ozarks where no mtDNA variation was detected in spotted salamanders, and two were sampled in the southern Ozarks where up to three haplotypes were detected per population (Phillips, 1994). We seined larvae from breeding ponds from early May through late September. Adults were taken from pitfall traps or seined from breeding ponds from January through April. We isolated

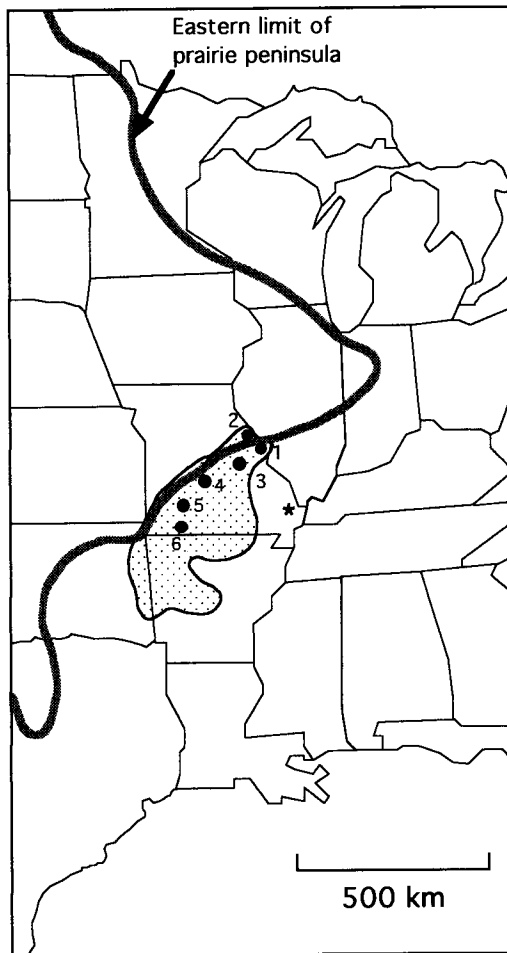


Fig. 1. Map of midwestern North America showing collecting localities for ringed salamanders (numbered dots; same as in Table 1), eastern limit of Prairie Peninsula, current range of the ringed salamander (stippled area), and location of the pollen analysis of King and Allen (1977; star).

total genomic DNA from nitrogen powdered tissue or whole blood. DNA was digested according to manufacturer's recommendations using the six-base recognizing restriction enzymes *BclI*, *BglII*, *EcoRI*, *EcoRV*, *KpnI*, *PstI*, *PvuII*, and *StuI* (New England Biolabs, Beverly, MA). Fragments of the digested DNA were separated by electrophoresis through agarose and the DNA was transferred to a support membrane by Southern blotting.

Southern blots were hybridized to radioactively labeled *A. maculatum* mtDNA clones pAm10 and pAm6 (Phillips, 1994). We visualized the DNA fragments using autoradiography. Restriction site information was inferred from fragment patterns using double digestion.

Permutation chi-square analysis using 1000 resamples was used to test for heterogeneity in haplotype frequencies among all populations and within and between the northern and southern populations. An arc-sine square-root test was used to test the null hypothesis that the two population groups have the same expected heterozygosity (the probability of drawing at random two different mtDNA haplotypes).

#### RESULTS AND DISCUSSION

The eight restriction enzymes produced 23 restriction sites. Only one *EcoRV* site and one *PstI* site were detected in the mtDNA of the ringed salamanders assayed. The other restriction enzymes cut the mtDNA from two to six times. The two variable enzymes, *PvuII* and *StuI*, cut two or three times and defined three haplotypes, AA, AB, BA (Table 1). The first position denotes the number of *PvuII* sites and the second the number of *StuI* sites (A indicates three, B indicates two).

The distribution of genotypes among populations and the frequencies within populations

TABLE 1. GEOGRAPHIC LOCATIONS, SAMPLE SIZES, AND MT DNA GENOTYPE FREQUENCIES OF RINGED SALAMANDER POPULATIONS. Codes are those used in Figure 1. The first letter of the haplotype designation refers to the number of *PvuII* sites, the second refers to the number of *StuI* sites (A = 3, B = 2).

Code	Population (latitude/longitude)	Sample size	Haplotypes (frequency)
1	St. Louis Co., MO (38°36.5'N-90°40.7'W)	20	AA (1.00)
2	Warren Co., MO (38°48.2'N-91°12.1'W)	18	AA (1.00)
3	Lincoln Co., MO (38°55.6'N-90°42.8'W)	23	AA (1.00)
4	Camden Co., MO (37°58.3'N-92°41.5'W)	12	AA (1.00)
5	Stone Co., MO (36°44.2'N-93°25.3'W)	14	AA (0.65) BA (0.35)
6	Stone Co., MO (36°43.7'N-93°26.0'W)	14	AA (0.50) AB (0.30) BA (0.20)

are given in Table 1. Only the southern populations (numbers 5–6) contained more than one haplotype. With the exception of population 4, these were also the sites with the smallest sample sizes. The northern populations (numbers 1–4) shared a single haplotype. There was heterogeneity in haplotype frequencies among all populations ( $\chi^2 = 46.89$ ,  $P = 0.00$ ) but not within the northern or southern populations (southern population:  $\chi^2 = 3.36$ ,  $P = 0.24$ ). Thus, the source of heterogeneity is the contrast between the northern and southern Ozarks. The expected heterozygosity was zero for the northern Ozarks and 0.56 for the southern Ozarks. The test statistic for the arc-sine square-root test was 7.6 indicating a highly significant difference in the level of genetic variation between the northern and southern populations.

The lack of mtDNA variation in the northern Ozark populations of the ringed salamander is predicted if populations recently colonized this area from the south. Under this hypothesis (Phillips, 1994), woodland salamanders along the southern border of the Prairie Peninsula were pushed south as grassland and savanna replaced forests in this direction during the Hypsithermal Interval, 8000 to 4000 years ago. As the climate ameliorated, the Prairie Peninsula contracted and forest replaced grassland. Woodland salamanders reinvaded from populations along the southern margin of the peninsula, but lineage sorting caused by waves of colonization and extinction reduced the mtDNA variation to one or a few common haplotypes from the source area (Nagyaki, 1976; Maruyama and Kimura, 1980; Hewitt, 1993). The effects of colonization and extinction would be expected to be more severe the farther north populations were established. An alternative, but not mutually exclusive, explanation is that the colonization was kin-structured and matrilineal (e.g., a group of related sisters), which led to a reduction of mtDNA diversity greater than that which would be expected for nuclear markers (Wade et al., 1994). Under either scenario, the Hypsithermal Interval occurred too recently for the accumulation of a substantial amount of genetic variation.

Phillips (1994) reviewed the literature on the climatic history of North America and cited paleoecological evidence to support this hypothesis. The most pertinent data came from a pollen analysis on sediments from peat deposits at a site in southeastern Missouri currently beyond the southeastern limit of the Prairie Peninsula (King and Allen, 1977). The data suggest that between 8700 and 5000 years ago, oak (*Quercus*) and ash (*Fraxinus*) pollen was replaced by pol-

len derived mainly from a grass-dominated community. This trend was reversed around 4000 years ago when the pollen profile returned to that of an oak- and hickory-dominated community. These changes were thought to reflect an expansion and retraction of the Prairie Peninsula in Missouri. It is possible that the ecotone between the two habitat types was dynamic, moving north and south as the climate fluctuated during the Hypsithermal. This situation would have compounded the effects of the colonization-extinction process and decreased the genetic variation of the colonizers at a faster pace than has been proposed (Nagyaki, 1976).

This study demonstrates the potential benefits of comparing biogeographical data across species and suggests that Holocene climate fluctuations may be an overlooked source of important historical effects for species in the midwestern United States, especially those not directly affected by Pleistocene glaciation.

#### ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant R01 GM31571 to ART. We are grateful to J. Brawn, G. Levin, and L. Page for comments which greatly improved this manuscript and to J. Phillips, E. Routman, O. Sexton, R. Wilkinson, R. Guffey, A. Gerber, and B. Schuette for help collecting specimens. A Wildlife Collecting Permit to CAP was provided by the Missouri Department of Conservation.

#### LITERATURE CITED

- BERNATCHEZ, L., AND C. C. WILSON. 1998. Comparative phylogeography of Nearctic and Palearctic fishes. *Mol. Ecol.* 7:431–452.
- BRAWN, J. D., T. M. COLLINS, M. MEDINA, AND E. BERMINGHAM. 1996. Associations between physical isolation and geographical variation within three species of Neotropical birds. *Ibid.* 5:33–46.
- HEWITT, G. W. 1993. Postglacial distribution and species substructure: lessons from pollen, insects and hybrid zones, p. 97–123. *In: Evolutionary patterns and processes*. D. R. Lees and D. Edwards (eds.). Academic Press, San Diego, CA.
- KING, J. E. AND W. H. ALLEN JR. 1977. A Holocene vegetation record from the Mississippi River Valley, southeastern Missouri. *Quat. Res.* 8:307–323.
- LARSON, A., D. B. WAKE, AND K. P. YANEV. 1984. Measuring gene flow among populations having high levels of genetic fragmentation. *Genetics* 106:293–308.
- MARUYAMA, T., AND M. KIMURA. 1980. Genetic variation and effective population size when local extinction and recolonization of subpopulations are frequent. *Proc. Natl. Acad. Sci. USA* 77:6710–6714.
- MERILA, J., M. BJORKLUND, AND A. BAKER. 1996. Ge-

- netic population structure and gradual northward decline of genetic variability in the greenfinch (*Carduelis chloris*). *Evolution* 50:2548–2557.
- NAGYLAKI, T. 1976. The decay of genetic variability in geographically structured populations. II. *Theor. Popul. Biol.* 10:70–82.
- PHILLIPS, C. A. 1994. Geographical distribution of mitochondrial DNA variants and the historical biogeography of the spotted salamander, *Ambystoma maculatum*. *Evolution* 48:597–607.
- SAGE, R. D., AND J. O. WOLFF. 1986. Pleistocene glaciations, fluctuating ranges, and low genetic variability in a large mammal (*Ovis dalli*). *Ibid.* 40:1092–1095.
- TEMPLETON, A. R., E. ROUTMAN, AND C. A. PHILLIPS. 1995. Separating population structure from history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* 140:767–782.
- WADE, M. J., M. L. MCKNIGHT, AND H. B. SHAFER. 1994. The effects of kin-structured colonization on nuclear and cytoplasmic genetic diversity. *Evolution* 48:1114–1120.
- WEBB III, T., AND R. A. BRYSON. 1972. Late- and post-glacial climatic change in the northern Midwest, USA: quantitative estimates derived from fossil pollen spectra by multivariate statistical analysis. *Quat. Res.* 2:70–115.
- WRIGHT JR., H. E. 1971. Late quaternary vegetational history of North America, p. 425–464. *In: Late Cenozoic glacial ages*. K. K. Turekian (ed.). Yale Univ. Press, New Haven, CT.
- (CAP) ILLINOIS NATURAL HISTORY SURVEY, CENTER FOR BIODIVERSITY, CHAMPAIGN, ILLINOIS 61820; AND (GS, ART) DEPARTMENT OF BIOLOGY, WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI 63130. PRESENT ADDRESS: (GS) UNIVERSIDAD CENTRAL DEL CARIBE, BAYAMON, PUERTO RICO 00960. E-mail: (CAP) [chrisp@mail.inhs.uiuc.edu](mailto:chrisp@mail.inhs.uiuc.edu). Send reprint requests to CAP. Submitted: 24 March 1999. Accepted: 21 Sept. 1999. Section editor: J. D. McEachran.