Biochemical Evolution in the Slimy Salamanders of the *Plethodon glutinosus* Complex in the Eastern United States

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ILLINOIS BIOLOGICAL MONOGRAPHS 57

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ILLINOIS BIOLOGICAL MONOGRAPHS **57**

UNIVERSITY OF ILLINOIS PRESS Urbana and Chicago

ILLINOIS BIOLOGICAL MONOGRAPHS

Volumes 1 through 24 contained four issues each. Beginning with number 25 (issued in 1957), each publication is numbered consecutively. Standing orders are accepted for forthcoming numbers. The titles listed below are still in print. They may be purchased from the University of Illinois Press, 54 East Gregory Drive, Champaign, Illinois 61820. Out-of-print titles in the Illinois Biological Monographs are available from University Microfilms, Inc., 300 North Zeeb Road, Ann Arbor, Michigan 48106.

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Manufactured in the United States of America
P 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

Highton, Richard.
Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern United States/Richard Highton, George C. Maha, and Linda R. Maxson.
p. cm.—(Illinois biological monographs; 57)
Bibliography: p.
Includes index.
ISBN 0-252-06007-5
1. *Plethodon*—United States—Evolution. 2. Slimy salamander—United States—Evolution. 3. Chemical evolution. 4. Amphibians—United States—Evolution. I. Maha, George C. II. Maxson, Linda R. III. Title. IV. Series.
QL668.C274H54 1989
597.6'5—dc19 88-5777
CIP

Contents

Abstract

Part 1. Geographic Protein Variation
RICHARD HIGHTON
Acknowledgments 2
Introduction 3
Materials and Methods 4
Results and Discussion 9
Summary 76

Part 2. Immunological Analysis of Geographic Variation in Plasma Albumins
GEORGE C. MAHA, LINDA R. MAXSON, AND RICHARD HIGHTON
Acknowledgments 80
Introduction 81
Materials and Methods 83
Results and Discussion 83
Summary 92

Literature Cited 93

Appendix 1: List of Localities 101

Appendix 2: Genic Variation and Heterozygosity Estimates in Samples of the *Plethodon glutinosus* Complex 105

Appendix 3: Immunological Distances of the *Plethodon glutinosus* Complex 145

Index 150

Abstract

Electrophoretic and immunological analyses of genetic variation within and among 135 populations from throughout the range of the *Plethodon glutinosus* complex suggest that it is comprised of 16 groups that have achieved the species level of divergence. Problems associated with taxonomically recognizing forms that are genetically, but not always morphologically, differentiated are discussed.

Part I
Geographic Protein Variation

Richard Highton

ACKNOWLEDGMENTS

I wish to thank the many students and colleagues who accompanied me on field trips to help obtain the material for this research. For information on localities, I thank the many herpetologists who shared their knowledge of local areas with me and sent specimens. I am also grateful for the essential laboratory help provided by many student assistants and graduate students and technicians, especially Ivette Aguirre, Debra Glover, Serap Oktay, and Jeffrey Streicher. Robert B. Peabody kindly provided data for some populations in the southern Appalachian Mountains and Robert Duncan for those in the Ouachita Mountains. Jerry A. Coyne, Arnold B. Grobman, Carla A. Hass, S. Blair Hedges, Allan Larson, Linda R. Maxson, and David B. Wake read the manuscript and provided many helpful suggestions. Thanks also are due to many state and federal agencies for issuing collecting permits and to the Computer Science Center and the General Research Board of the University of Maryland and the National Science Foundation (Grants GB-37320X, DEB-76-10583, DEB-79-03858, DEB-81-17983) for financial support.

INTRODUCTION

Modern methods of population genetic analysis make it possible to evaluate geographic variation in species and to analyze the patterns of their genetic substructuring. The slimy salamander, *Plethodon glutinosus* (Green, 1818), ranges from southwestern New England west to Missouri and south to Florida and Texas. A previous attempt to analyze patterns of geographic variation in the species (Highton, 1962b) was unsuccessful because of the paucity of variable morphological characters. The present electrophoretic study of geographic variation in 22 presumed genetic loci in 135 populations has provided the data needed to analyze variation in the species. The results indicate that the *P. glutinosus* complex, until recently considered a single taxonomic species, consists of 16 divergent units that are evolutionarily close to, or already have achieved, species status.

Two pairs of sympatric, reproductively isolated species, so similar to *P. glutinosus* in standard taxonomic characters that they had been included in that taxon (Highton, 1962b), occur in two different regions. Widely distributed on the Cumberland Plateau is a form previously named *P. kentucki* (Mittleman, 1951). It is sympatric with *P. glutinosus* and its taxonomic validity was demonstrated by Highton and MacGregor (1983) and Maha *et al.* (1983). A second sibling species, *P. aureolus* Highton (1984), occurs in southeastern Tennessee and southwestern North Carolina and is sympatric throughout its range with another member of the *P. glutinosus* complex, *P. teyahalee* Hairston (1950), which was also recognized as a full species by Highton (1984). He found that *P. aureolus*, *P. teyahalee*, and *P. glutinosus* are sympatric at a site in Polk County, Tennessee, with apparent reproductive isolation among all three forms. Data for salamanders from the latter site are not included in this study of 135 populations. The study includes data for 2 sympatric, non-interbreeding species found at 6 of the 129 localities: *P. glutinosus* and *P. kentucki* at 4 sites, and *P. aureolus* and *P. teyahalee* at 2 sites.

This paper is one of a series of studies on electrophoretically detectable genetic variation in salamanders of the genus *Plethodon* (Highton and Webster, 1976; Highton, 1977; Larson and Highton, 1978; Duncan and Highton, 1979; Highton and Larson, 1979; Highton and MacGregor, 1983; Highton, 1984; and Wynn, 1986).

MATERIALS AND METHODS

An attempt was made to obtain 25 to 30 individuals from each of the 129 sites (up to 5 hectares of continuous woodland per site) scattered throughout the range of the species (Fig. 1 and Appendix 1). It was not possible to obtain the full number from 8 of the localities, but at least 10 salamanders were collected from all of them. Genetic variation is low in the populations with smaller sample sizes, so genetic distance estimates are probably not affected significantly. Two separate species were represented at 6 of the sites, therefore additional material was obtained to bring the sample sizes of both forms up to an effective number. Large sample sizes are needed to estimate accurately the geographic patterns of allele frequency variation in polymorphic loci.

Populations were selected to include (1) sites in all of the physiographic provinces in which the complex is known to occur, (2) material from or near type localities of all species or subspecies presently recognized or synonymized under the name *P. glutinosus*, (3) several populations of each eastern geographic variant described by Highton (1970, 1972), and (4) sites where sufficient numbers of salamanders could be obtained for a satisfactory analysis of genetic variation. Electrophoretic data are available from over 200 additional populations. Although sometimes based on smaller sample sizes or fewer than 22 loci, or both, these data have been useful in better delineating geographic ranges of groups detected in populations from the 129 localities for which we have more complete data.

Genic variation was analyzed in 22 presumed independent genetic loci using the protein-buffer combinations listed in Table 1. These are the same loci used by Highton and MacGregor (1983) and Highton (1984). The genetic data are listed in Appendix 2, except for one general protein locus (Pt-3) that is monomorphic in all samples.

Salamanders were brought to the laboratory alive and their blood was centrifuged to obtain plasma samples. Blood albumin was scored on 7

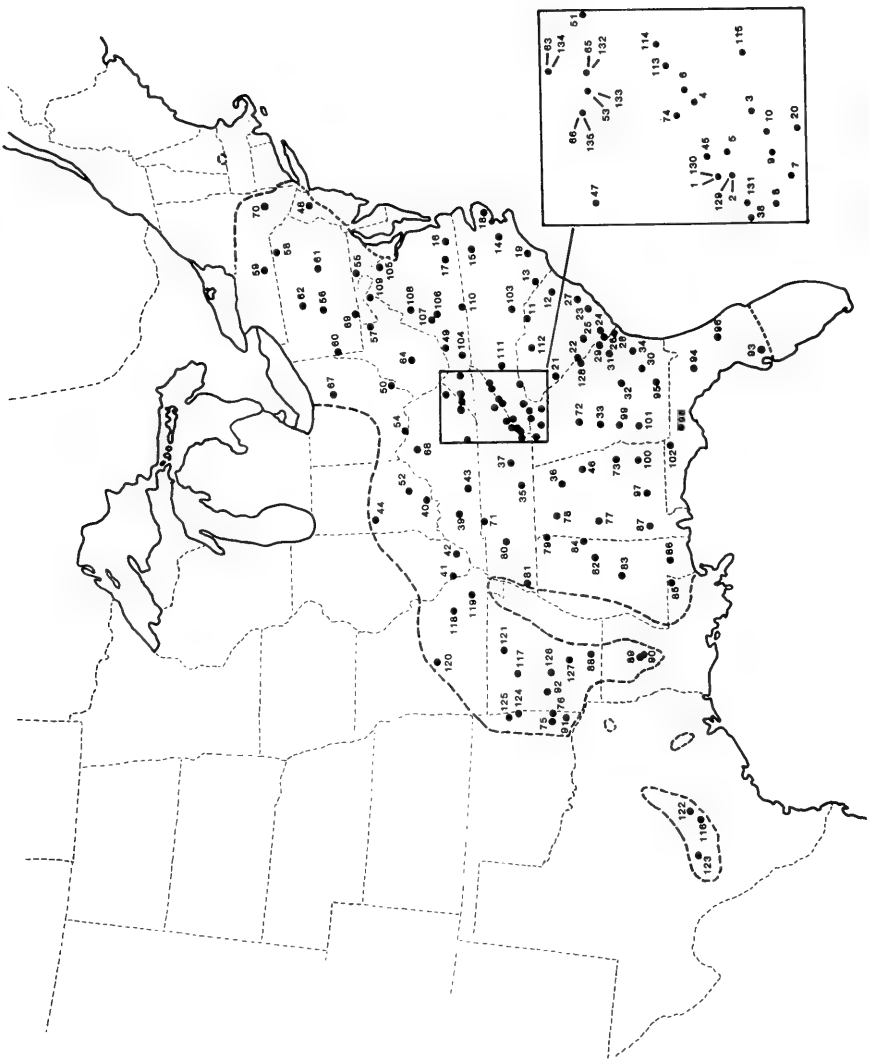

Fig. 1. Sites of the 135 samples from 129 localities in the range of the *Plethodon glutinosus* complex in the eastern United States.

percent acrylamide disc gels and transferrin on 10 percent gels using the method of Davis (1964). Whole animals were homogenized and then centrifuged to obtain aqueous extracts of proteins, which were stored below −70°C until utilized for electrophoresis. Electrostarch and Sigma starch were employed using the methods of Brewer (1970), Selander *et al.* (1971), and Shaw and Prasad (1970). When two loci were associated with a protein, the more anodal locus was assigned the numeral "1" and the more cathodal was designated "2." Allelomorphs, hereafter referred to as alleles, were designated alphabetically by order of electrophoretic mobility, with "a" being the most distant from the origin.

Table 1. Buffers used in electrophoresis and proteins assayed on each buffer.

Buffer	Assay
Tris Versene Borate	α–Glycerophosphate Dehydrogenase (α–Gpd), Glutamate Dehydrogenase (Gdh), General Protein (Pt-1), 6-Phosphoglutonate Dehydrogenase (6-Pgd), Phosphoglucose Isomerase (Pgi)
Lithium Hydroxide	Esterase (Est), Leucine Aminopeptidase (Lap), Peptidase (Pep), General Protein (Pt-2), Phosphoglucose Isomerase
Poulik	Esterase, Fumarase (Fum), Lactate Dehydrogenase (Ldh), General Protein (Pt-3)
Tris-Citrate pH 6.7	Glutamic Oxaloacetic Transaminase (Got), Lactate Dehydrogenase, Malate Dehydrogenase (Mdh), Phosphoglucomutase (Pgm)
Tris-Citrate pH 8.0	Glutamate Dehydrogenase, Glutamic Oxaloacetic Transaminase, Isocitrate Dehydrogenase (Idh), Leucine Aminopeptidase
Tris-Glycine (disc)	Albumin (Alb), Transferrin (Trf)

Unless otherwise indicated, estimates of genetic similarity are expressed as Nei's *I*, the normalized identity of genes, and genetic distance is expressed by Nei's standard genetic distance, *D* (Nei, 1972). These measures were calculated from a computer program prepared and

furnished by Nei and Roychoudhury (1974). Other measures of genetic similarity and distance were calculated by a modified version of the BIOSYS-1 program of Swofford and Selander (1981), which they provided. Estimates of genic heterozygosity (*H*), the mean proportion of loci heterozygous per individual, were estimated from allele frequencies. Phenograms and distance Wagner trees were obtained by using the BIOSYS-1 program and by the Taxan program made available by Rita R. Colwell of the University of Maryland.

This study, comparing alleles in 22 loci (some highly variable) in almost 4000 animals, took 8 years to complete. Because several technicians and students assisted in the laboratory work and several different starch lots were used during the course of the research, the chances for error may be higher than in previous studies. However, samples of the homogenates of all animals were stored at or below −70°C for the entire study so that every allele in all populations was run side by side with standards on comparison gels. A few populations (indicated in Appendix 2) had rare alleles that could not be compared with all standards because all of the sample had been depleted in previous runs.

Two samples that were originally to be included in the study were omitted when it was concluded that they may represent hybrid populations. The data have been transferred to other studies of hybrid zones among the various groups of the *P. jordani* and *P. glutinosus* complexes.

For many of the populations the sample sizes for the two blood protein loci (albumin and transferrin) are much larger than those for the remaining loci because data were available from an earlier unpublished study of geographic variation in these two loci.

Exact localities and elevations for the collection sites are presented in Appendix 1 and are shown in Figure 1. The samples are numbered in the order of their clustering on the UPGMA phenogram of *I* values (Fig. 2). This phenogram is the only clustering procedure that includes all 135 populations. Only 115 samples were used for the other tree-building methods.

As in most electrophoretic studies, some genetic variation is probably present that could not be resolved satisfactorily in all individuals. For example, it was not possible to separate the albumins of groups 3 and 6 from the middle Atlantic states, although in a 1969 unpublished study, Virginia Maiorana had consistently been able to distinguish the albumins of these groups by using the method of Smithies (1959). Thus variation in allele *d* of the Alb locus is obviously more complex than

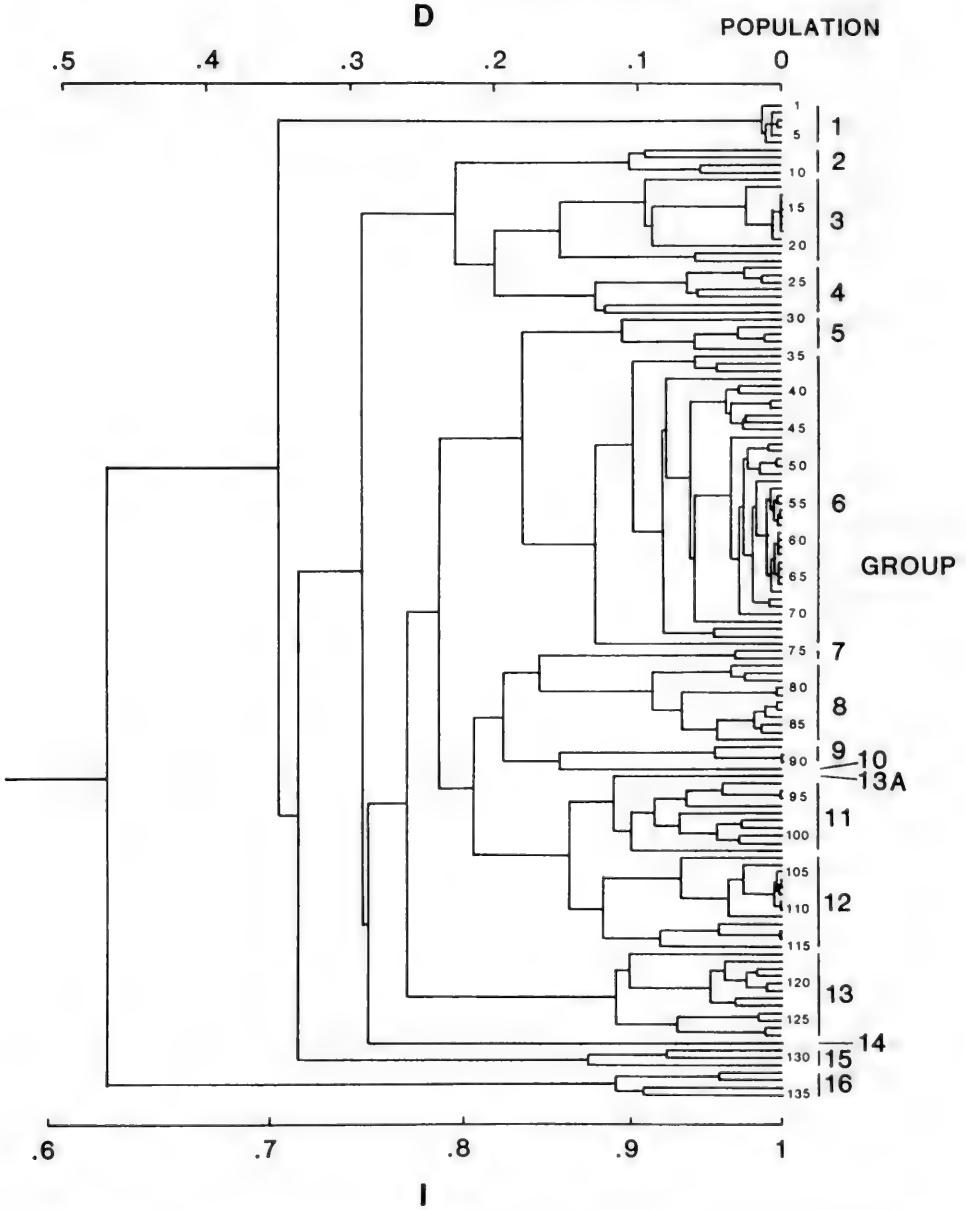

Fig. 2. UPGMA phenogram of Nei's *I* values. Sample 92 is indicated as being a member of Group 13 by the symbol "13A." Populations are numbered as in Figure 1 and Appendix 1.

indicated here. Other similar examples are probably alleles *t* of the Est locus, *b* of the Gdh locus, *b* of the Idh-1 locus, *b* of the Mdh-1 locus, *d* of the Pep locus, *c* of the Pt-2 locus, and *j* of the Trf locus. Allele *jj* of the Est locus and allele *h* of the Pt-2 locus are null alleles. Allele *e* of the Pt-2 locus is not present in any of the 135 populations of this study but is common in related species (Table 4).

Additional specimens from most localities are preserved in the writer's collection and will be deposited in the National Museum of Natural History (USNM) collection.

RESULTS AND DISCUSSION

Grouping of Populations

Populations previously recognized as a single species, *P. glutinosus* (Appendix 2 and Fig. 2), have been found to have substantial genetic differentiation. Three different species of the complex (*P. kentucki, P. aureolus*, and *P. teyahalee*) are sympatric with *P. glutinosus* and there is little or no evidence of hybridization among them (Highton and MacGregor, 1983; Highton, 1984).

A number of populations are very closely related genetically with *D* values under 0.025 ($I > 0.975$, Fig. 3). Many of these populations are from the northern parts of the range and are probably derived from different source populations that dispersed northward when the last glaciers receded, as discussed below. Several populations from the Gulf Coastal Plain physiographic province just east of the Mississippi River are also closely related to each other. Most other adjacent populations have Nei genetic distances higher than 0.025.

Arranging the populations into genetically related groups facilitates a discussion of their relationships. A number of divergent groups of populations are genetically quite homogeneous internally and are geographically cohesive (Fig. 4).

Some major differences in the arrangement of groups are seen in the UPGMA phenograms and distance Wagner trees which use different measures of genetic similarity and distance (see below). Apparently, there is no *a priori* method of determining which of the various tree-building methods would provide a dendrogram that best indicates the evolutionary relationships of populations from biochemical data (see Nei *et al.*, 1983, for a discussion of the problem). However, the various

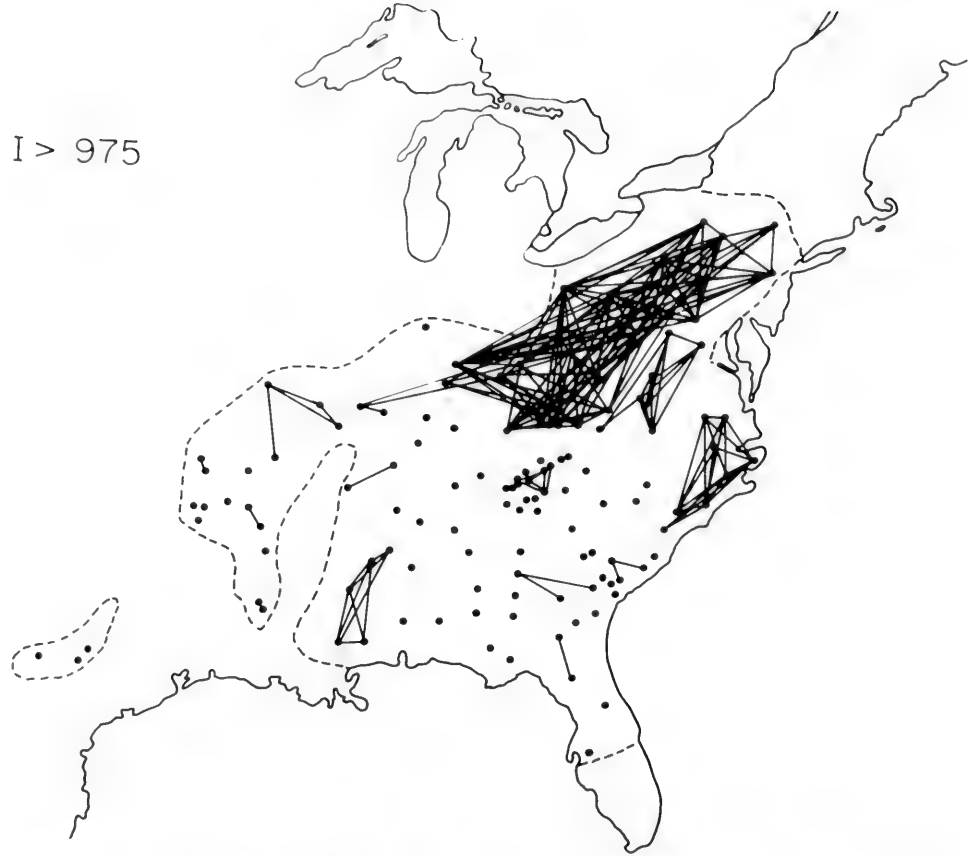

Fig. 3. Lines connect populations of the *Plethodon glutinosus* complex that have $I \geq 0.975$. Samples 129–131 (*P. aureolus*) and 132–135 (*P. kentucki*) are omitted.

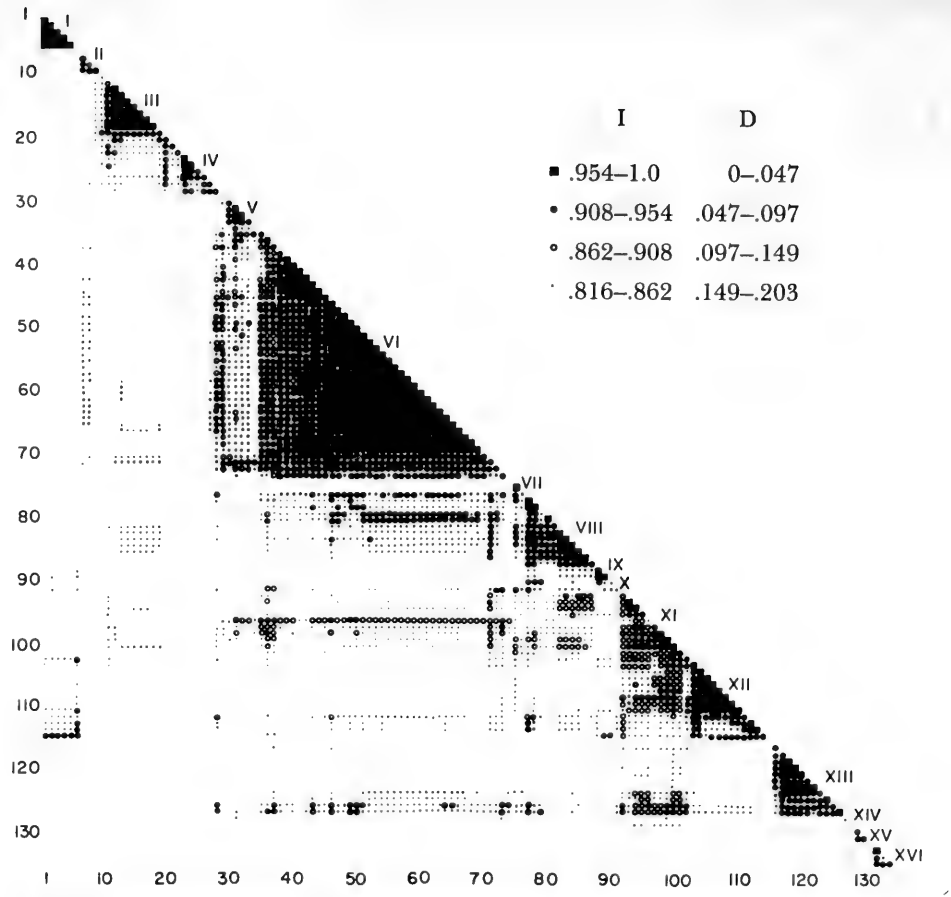

Fig. 4. Graphic representation of the genetic relationships among all 135 populations. Roman numerals indicate the 16 groups.

methods may suggest phylogenetic reconstruction hypotheses. There is also considerable information on morphological variation in the *P. glutinosus* complex (Highton, 1962b, 1970, 1972) that helps to interpret the genetic data.

Any level of *D* chosen to separate populations into taxonomic groups is arbitrary. The lower the value of *D* selected, the greater the number of groups, and *vice versa*. However, there seems to be a level of divergence ($D = 0.15$) that groups the samples into units that not only are geographically continuous but also are not morphologically divergent within groups. If a higher mean *D* level is used for determining the group limits, populations that are geographically separated or morphologically divergent, or both, are sometimes included in the same group. If a lower value of *D* is used, several groups that appear morphologically similar are subdivided into units that are not geographically contiguous. Using a *D* level of 0.15 *after* studying the results is, of course, subjective and others might favor different methods of grouping (or might not group populations by using genetic distance data). However, genetic data obtained by electrophoretic evaluation of 20 to 30 protein loci has clarified our understanding of the relationships of populations in many plethodontid salamander species (see Wake, 1981, for a review of the literature). The discovery of the clocklike nature of substitution rates in the proteins evaluated in electrophoretic studies (see Sarich, 1977; Wilson *et al.*, 1977, 1987) provides a strong basis for using genetic data in the analysis of evolutionary relationships of populations. If the amount of time that populations have diverged is correlated with a build-up of genetic divergence, then genetic distances should provide better estimates of phylogenetic relationships than does morphology. Morphology is well known to have evolved at highly variable rates (Simpson, 1953; Wake, 1981; Larson, 1984). Electrophoretic, immunological, and DNA hybridization data in *Plethodon* are in good agreement (Mizuno and MacGregor, 1974; Highton and Larson, 1979; Maxson and Maxson, 1979), and support the hypothesis that independent biochemical methods consistently reveal evolutionary relationships among taxa. Morphological studies have generally provided less satisfactory results, and several investigators have suggested different arrangements of the species of *Plethodon* into species groups (Highton, 1962b). If divergence time is also correlated with the evolution of reproductive incompatibility (Mayr, 1942, 1963), then taxonomic decisions involving allopatric populations at the species level

might more likely be correct if genetic distance data are available.

Baverstock *et al.* (1977) and Thorpe (1982) also have argued in favor of using a threshold of about 15 percent genetic differentiation as the amount of genetic divergence necessary for the recognition of allopatric populations as species. Baverstock *et al.* (1977) suggest that if two allopatric populations possess fixed electrophoretic differences at 15 percent or more of their loci, then it is probable that they belong to different biological species. (Two populations that have fixed differences at 15 percent of their loci would have an $I < 0.85$ or a $D > 0.16$.) In a survey of the literature (excluding birds), Thorpe (1982) found that 97 percent of I values between species are below 0.85 and that within species 98 percent of I values exceed 0.85.

In this study there are two borderline cases. On the UPGMA phenogram (Fig. 2), the mean D for the 130 comparisons between samples 93–102 and 103–115 is 0.146. Because these two groups represent morphologically different populations from isolated geographic areas, they are regarded as separate groups (see below). The mean D value of 20 comparisons between samples 11–20 and 21–22 is 0.155. Because these populations are all morphologically similar and come from contiguous areas, they are regarded as belonging to the same group.

Of 1,198 within-group comparisons, 55 (4.5%) have D values above 0.15. Fourteen of these involve comparisons of samples 21 and 22 with other members of Group 3, and nine are comparisons of sample 92 with other members of Group 13. Of 7,847 between-group comparisons, 444 (5.7%) have D values below 0.15.

The allele frequency data in Appendix 2 show a variety of differences among the groups. In about half the cases there are fixed differences in at least one locus between parapatric and sympatric groups so that all individuals of one group are distinguishable from all those of the other. In the remaining cases, loci that differentiate groups have only allele frequency differences. For example, the Gdh locus is the highest contributor to the differentiation of the parapatric groups 5 and 6 (mean $D = 0.18$). Allele *a* has a high frequency in all populations of Group 5 and is fixed in 3 of 5 samples, whereas allele *b* has a high frequency in Group 6 populations and is fixed in 38 of 40 samples. However, one sample from Georgia of Group 6 (72) has a 0.38 frequency of allele *a* and two Group 5 samples (31 and 32) have frequencies of allele *b* of 0.24 and 0.23, respectively. Thus over 99 percent of the sala-

manders of Group 6 are different at the Gdh locus from over 90 percent of those in Group 5, but three populations have a substantial frequency of alleles from the other group. In some loci, for example the Pt-2 locus, which usually distinguishes the parapatric groups 6 and 13 (mean *D* between the two groups, 0.24), there is a difference between all 41 samples of Group 6 (fixed for allele *a*) and 10 of 12 samples of Group 13 (polymorphic for alleles *f* and *h*), but 2 samples (126 and 127) of Group 13 are fixed for allele *a*. A third example is more complex. The sympatric species *P. teyahalee* (Group 1) and *P. aureolus* (Group 15) have major frequency differences at six loci and the mean *D* between the two groups is 0.42, but always at least one population of one or both species has in low frequency an allele characteristic of the other species. This phenomenon might be due to ancient polymorphisms with the alternate rare alleles still occurring in only a few living descendants of the common ancestor, rare hybridization between the two species, or hybridization of *P. aureolus* with Unicoi Mountain *P. jordani*, which at some loci has a high frequency of alleles also found in *P. teyahalee* (Highton, 1984).

An UPGMA phenogram (Sneath and Sokal, 1973) of Nei's *I* values of all 135 samples (Fig. 2) indicates a high level of genetic divergence among groups, not only of the four species already recognized but also in the remaining populations still assigned to *P. glutinosus*. Using the level of $D = 0.15$ for grouping populations, 16 major groups are indicated in the phenogram. Because the UPGMA clustering algorithm assumes equal rates of molecular evolution on all lines, an assumption which may not necessarily be correct, the evolutionary relationships of these groups will be interpreted below, after discussion of the distance Wagner trees (Farris, 1972).

Group 1 (populations 1–6). Samples represent *P. teyahalee* Hairston (1950), already recognized by Highton (1984) as a distinct species because it occurs sympatrically with Group 15 (*P. aureolus*) at 28 known localities and with Group 6 (*P. glutinosus*) at a single site in Polk County, Tennessee, with little or no indication of hybridization. Adult salamanders of this form may be distinguished from those of other groups by their large size and very small white dorsal spots. There is overlap with variation in this pair of traits only with populations of Group 12, but the latter usually have much larger white dorsal spots. Salamanders in Group 1 sometimes have very small red spots on the legs. Mor-

phologically, groups 1 and 12 are primarily distinguished by the size of the white dorsal spots, but genetically, they are quite different (see below on the possible origin of *P. teyahalee* through hybridization of Group 12 and *P. jordani*). *P. teyahalee* occurs in the Blue Ridge physiographic province of North Carolina and in immediately adjacent areas of Tennessee and South Carolina west of French Broad River. In South Carolina it also enters the Piedmont physiographic province. The distribution of this form is shown in Figure 8 and in Highton (1970: Fig. 5; 1984). There is very little geographic genetic variation throughout the range of this group.

Group 2 (populations 7–10). Samples of populations 7, 9, and 10 belong to a form from the mountains of northeastern Georgia that is unusual in often lacking the dorsal spots so characteristic of almost all other populations of the *P. glutinosus* complex (except those from Jasper County, South Carolina, which also lack the lateral spots). Salamanders in Group 2 usually have abundant lateral white or yellow spotting. The distribution of this phenotype was mapped in Highton (1970: Fig. 5). Surprisingly, a nearby sample of salamanders with abundant brassy dorsal spotting (population 8) is also a member of this group genetically. Although Hairston and Pope (1948) assigned some specimens of this form to their *P. jordani rabunensis* Pope and Hairston (1948), (see also Hairston, 1950), the salamanders of Group 2 from northeastern Georgia are genetically different from the topotypic Rabun Bald population that is correctly assigned to *P. jordani* (Peabody, 1978). Therefore no name is now available for this group.

Group 3 (populations 11–22). Samples of populations 12–16, 18, and 19 are from the Coastal Plain physiographic province of Virginia, North Carolina, and northeastern South Carolina. Population 17 is from the eastern Piedmont physiographic province of Virginia. The range of this group also extends into the Piedmont of South Carolina (population 11) and population 20 is from Habersham County in northeastern Georgia. The Virginia populations of this form were recognized as distinct by their small size and very small, brassy dorsal spots (Highton, 1972) and were referred to as the "Coastal Plain type." The North Carolina populations are similar in appearance. The northern populations (13–19) are virtually identical to each other genetically and have little genetic variation. Most populations from South Carolina have

more abundant lateral white or yellow spotting than do those from North Carolina and Virginia. The southwestern populations have larger, brassy dorsal spots (Highton, 1962b). Samples of populations 21 and 22 are differentiated genetically from the remaining members of the group ($\bar{D} = 0.155$). The type locality of *Plethodon glutinosus chlorobryonis* Mittleman (1951), at or near locality 14, is within the range of this group. The name *Salamandra cylindracea* Harlan (1825) probably should be assigned to a form represented by the population near Camden, South Carolina (Highton, 1962b). If Harlan's description was based on animals from south of Camden, he probably was describing this form, but populations north of Camden are of Group 12 (see below).

Group 4 (populations 23–29). Samples of populations 23–27 from the Coastal Plain physiographic province of southern South Carolina and two others from adjacent coastal Georgia just west of the Savannah River (28 and 29) are members of this group. Populations 24 and 26 represent the unspotted morphotype from Jasper County, South Carolina, described by Neill (1948). The same phenotype also occurs as a common to rare variant in other South Carolina populations. Spotted animals of Group 4 are similar in appearance to southwestern populations of Group 3. The old name *Salamandra variolata* Gilliams (1818), with type locality "southern States," is available for Group 4 if Schmidt's (1953) restriction of the type locality to the vicinity of Charleston, South Carolina, is accepted. However, Gilliams (1818) stated that the specimens on which the description was based were received from the "Florida Party." If they actually came from Florida, the name would not apply to this group but rather to Group 11. According to Weiss and Ziegler's (1931) account of Thomas Say's trip to Florida, Say might have collected the type specimen in South Carolina, the Georgia Sea Islands, or along the St. Johns River in Florida, or even on his overland trip from Philadelphia to South Carolina. There is nothing in the original description to aid in assigning the name to any of the *P. glutinosus* complex groups.

Group 5 (populations 30–34). Samples of this group are from east-central Georgia. Salamanders of population 33, from the Piedmont physiographic province, are large in size, but the other four samples are from the Coastal Plain physiographic province and are smaller in size.

They have brassy dorsal spots but often fewer than do most other spotted *P. glutinosus*. No name is available for this form.

Group 6 (populations 35–74). This group occurs over a wide area from eastern Alabama and northwestern Georgia north to Illinois and east to West Virginia, western Virginia and Maryland, Pennsylvania, New Jersey, New York, and southwestern Connecticut. The distribution of this form in the middle Atlantic states (Pennsylvania, Maryland, Virginia, and West Virginia) corresponds to that of the "brassy-spotted type" whose range was mapped by Highton (1972). Salamanders of most populations are large sized with abundant, large, brassy dorsal spots and have dark chins, compared to sympatric light-chinned, smaller *P. kentucki* (Highton and MacGregor, 1983). Populations from eastern Kentucky northeast to New York (47–70) are all very similar to one another genetically. Other western and southern populations show much more local genetic differentiation. The name *Salamandra glutinosa* Green (1818), with type locality Princeton, New Jersey, clearly refers to this form. The isolated record from southern New Hampshire (Highton, 1963) most probably belongs to this group.

Group 7 (populations 75–76). Samples are from Round and Kiamichi mountains, Oklahoma, and represent a type whose genetic distinctness has already been noted by Duncan and Highton (1979). The sample from locality 8 of their study represents the same population as that of locality 76 of the present study (although different individuals are used here). Group 7 appears indistinguishable morphologically from other nearby populations except that in Group 7 the melanophore pigmentation on the chin is often reduced compared to the pigmentation on adjacent populations of Group 13. Because of the lighter chins of Kiamichi Mountain *P. glutinosus*, Blair and Lindsay (1965) suggested that this group hybridizes with sympatric *P. ouachitae*, but Duncan and Highton (1979) could find no genetic evidence to support this hypothesis. No name is available for this form.

Group 8 (populations 77–87). In the Coastal Plain physiographic province of western Kentucky and Tennessee, western Alabama, Mississippi, and the Florida parishes of Louisiana, there is a small-sized form represented by population samples 77 and 79–87. It has no distinguishing morphological features and often resembles Group 11 in appear-

ance. Sample 78 is from the Appalachian Plateau physiographic province of northern Alabama and although adults attain a larger size than those of the Coastal Plain populations, it is genetically similar to them and is assigned to this group. No name is available for this form.

Group 9 (populations 88–90). Samples from the Coastal Plain physiographic province of central Louisiana and southern Arkansas are small in size and have distinctive dorsal coloration: large dorsal spots with diffused brassy pigment. No name is available for this form.

Group 10 (population 91). The sample from southeastern Oklahoma is closest genetically to Group 9. In size and color pattern, however, it is similar to other nearby populations of groups 7 and 13. No name is available for this form.

[Although sample 92 clusters with 10 samples from the southeastern Coastal Plain physiographic province (Group 11) in the UPGMA phenogram, it is closer genetically to samples of populations 126 and 127 of Group 13 (D = 0.08–0.09) than to any of the samples of Group 11. Therefore, this population is regarded as a member of Group 13 rather than of Group 11 and is shown as Group 13A in Fig. 2.]

Group 11 (populations 93–102). Populations 93–102 from the southeastern Coastal Plain physiographic province of Florida, southern Georgia, and southern Alabama are characterized by their small size and large, brassy dorsal spots. The name *Plethodon glutinosus grobmani* Allen and Neill (1949) is available for this form if the older name *Salamandra variolata* Gilliams is properly applied to another form (see discussion under Group 4).

Group 12 (populations 103–115). Samples of these populations are characterized by their large size and large white dorsal spots. This group ranges from the French Broad River in western North Carolina and eastern Tennessee northeast through most of the uplands of western Virginia. It also occurs in the panhandle of northeastern West Virginia and in parts of the Coastal Plain physiographic province of eastern Virginia. The distribution of this "white-spotted type" in Virginia and West Virginia was mapped in Highton (1972). The northern populations of this group (105–110) are genetically almost identical with one another. The name *Salamandra cylindracea* Harlan

(1825) is available for this form, if it is based on Group 12 animals from north of Camden, South Carolina (see discussion of this name under Group 3).

Group 13 (populations 92, 116–127). This group includes all the remaining samples from west of the Mississippi River. As discussed above, sample 92 is also regarded as a member of this group. Group 13 includes all the samples from Missouri and Texas and those from northeastern Oklahoma and northern and central Arkansas. No specimens from the two small areas where *P. glutinosus* occurs in the Coastal Plain physiographic province of eastern Texas are available, so the genetic relationships of these populations are unknown. Populations of *P. glutinosus* from the Ozark Plateau and Ouachita Mountains are large, dark-chinned salamanders with large, brassy dorsal spots and closely resemble those of Group 6. The sample of population 127 from the Coastal Plain of southern Arkansas is probably similar, although too few animals have been collected there to accurately estimate the maximum size. In contrast, salamanders of the Texas populations appear to have a lighter ground color and smaller spots. Grobman (1944) recognized the latter as a subspecies, *Plethodon glutinosus albagula*, diagnosed by its lighter chin, but Highton (1962b) noted that some Texas populations have dark chins and that the eastern white-spotted populations (groups 1 and 12) also have light chins, as do groups 2, 15, and 16. Two of the three Texas samples (122 and 123) are of the dark-chinned form and the other (116) is of the light-chinned type. The dark- and light-chinned populations in Texas appear to be differentiated genetically ($D = 0.09$ and 0.13 between sample 116 and the other two). Indeed, the ten comparisons of Texas samples 122 and 123 with samples 117–121 (from Arkansas and Missouri) show that the former have lower genetic distances ($\bar{D} = 0.05$) from the latter than they have from sample 116. Highton (1962b) suggested that the Edwards Plateau of Texas might have been invaded twice by *P. glutinosus* populations from the uplands of Oklahoma and Arkansas, and that the earlier invasion might have evolved into the light-chinned form in Texas. The later invasion by animals similar to the present populations of Arkansas and Oklahoma is now represented by the dark-chinned populations in Texas. The genetic data are consistent with this hypothesis. However, all these populations are similar enough genetically to be included in one group, for which the name *P. albagula* is available.

Group 14 (population 128). This population from the upper Coastal Plain physiographic province of Richmond County in eastern Georgia is distinct genetically from all other populations. It also has unusual pigmentation; the dorsal spots have much less brassy flecking than do spots of nearby populations of several other groups. No name is available for this form.

Group 15 (populations 129–131). These samples represent a distinct species, *P. aureolus* Highton (1984). It is sympatric with *P. teyahalee* throughout its range and is also sympatric with *P. glutinosus* (Group 6) at a single known locality in Polk County, Tennessee, with little or no evidence of hybridization with either form. This small-sized species has abundant large, brassy dorsal spots.

Group 16 (populations 132–135). This group represents a distinct species, *P. kentucki* Mittleman (1951), as demonstrated by Highton and MacGregor (1983). Its albumin has been shown to be immunologically distinct by Maha *et al.*, (1983). This small species has a light chin and fewer, smaller, and less brassy dorsal spots than has sympatric *P. glutinosus* of Group 6. It occurs in the Cumberland Plateau of eastern Kentucky, northeastern Tennessee, southwestern Virginia, and western West Virginia. The four populations included here (from Harlan and Pike counties, Kentucky, and Wise County, Virginia) are different from some of the other samples evaluated genetically by Highton and MacGregor (1983). Three from their study (two from Summers County, West Virginia, and one from Dickenson County, Virginia) are especially divergent ($\bar{D} = 0.30$) to the other samples of *P. kentucki*. Because I have not been able to obtain adequate samples of *P. kentucki* from these eastern localities, they are not included in this study. However, it appears from the few data available that *P. kentucki* may be divisible into two forms at the level used here to separate groups.

The distribution of groups 1 to 14 and the mean Nei genetic distances between parapatric groups are shown in Figure 5.

Reconstruction of phylogenies from electrophoretic data is subject to many possible sources of error: laboratory errors in scoring gels, sampling errors in the collection of specimens and in the selection of loci to be evaluated, the partly stochastic nature of the evolutionary process, convergence of band mobilities of different genotypes, the possibility that natural selection might have a stabilizing or a balancing effect on

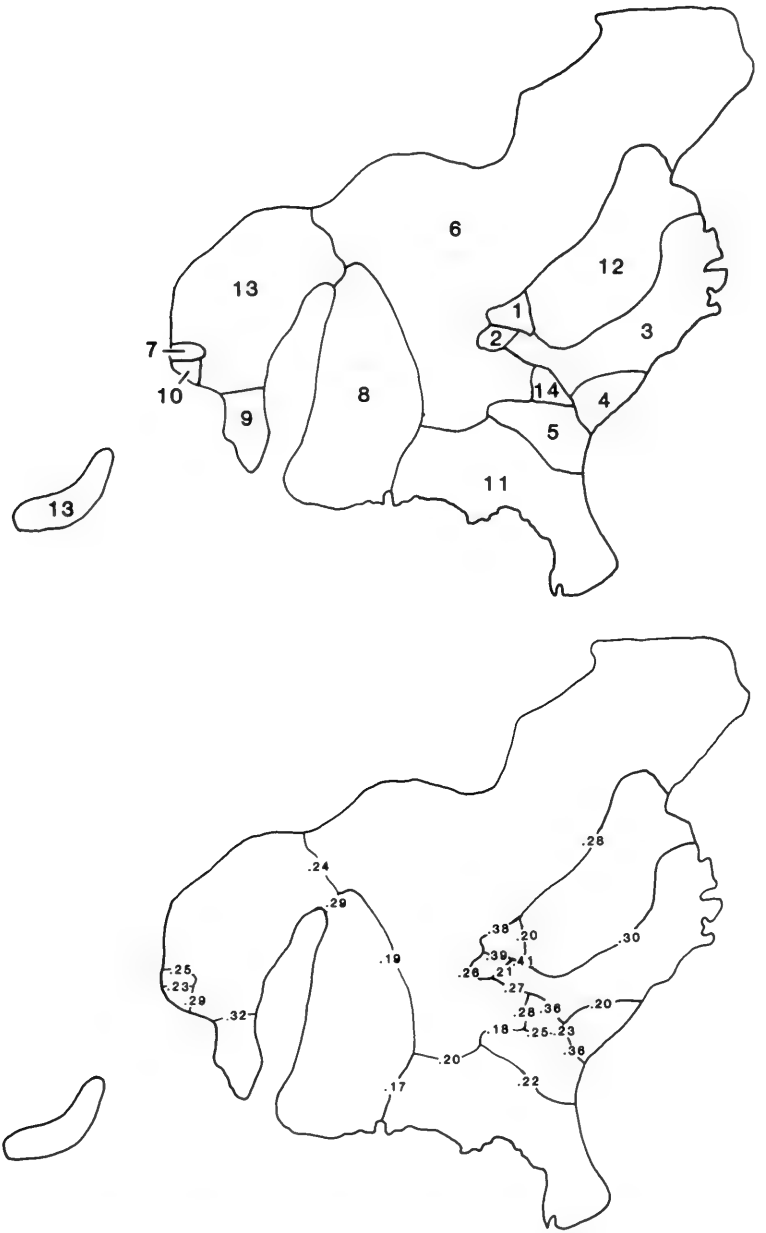

Fig. 5. *Top.* Numbers of the groups. Groups 15 (*P. aureolus*) and 16 (*P. kentucki*) are omitted. *Bottom.* Mean genetic distances (Nei's *D*) between parapatric groups.

some loci, the nonmetricity of Nei coefficients, the likelihood of gene flow through hybridization between some groups, and the assumption of the UPGMA method of equal evolutionary rates along all lines. In light of these possibilities for error, it is remarkable that only one population (92) appears, on the basis of geography, misplaced on the UPGMA phenogram.

Farris (1981) has argued strongly against the use of UPGMA phenograms of Nei's *I* or *D* values for making phylogenetic reconstructions. Not only are the phenograms constructed on the assumption of equal rates of molecular evolution, but Nei distances are not metric and therefore may violate the triangle inequality. Farris suggests that there are conceptual problems with all of the available distance measures commonly used by those studying molecular evolution. On the other hand, Tateno *et al.* (1982) and Nei *et al.* (1983) used computer simulations to study the accuracies and efficiencies of different methods for constructing phylogenetic trees from molecular data under the assumption of equal rates of evolution. Although they found that no genetic distance measure or clustering method was consistently superior, in general the topologies obtained by the UPGMA method using Nei distances were recommended. Felsenstein (1984) has also defended the use of genetic distance measures in inferring phylogenies because they may represent the expected rather than the actual amount of change.

Because of the very high cophenetic correlation coefficients obtained for phenetic clustering dendrograms in previous electrophoretic studies on *Plethodon* (Highton and Webster, 1976: $r = 0.97$; Larson and Highton, 1978: $r = 0.99$; Duncan and Highton, 1979: $r = 0.95$; Highton and Larson, 1979: $r = 0.97$; and Highton and MacGregor, 1983: $r = 0.97$), it appears that the data provide consistent estimates of genetic relationships among groups. It is possible, however, that the high cophenetic correlation coefficients obtained in the above studies were partly due to the fact that some of the comparisons involved relatively divergent forms. A phylogenetic tree constructed by the method of Fitch and Margoliash (1967) for the data in Highton and Larson (1979) resulted in a tree very similar to that in the UPGMA phenogram. Summing all branch lengths from the base of the tree (Highton and Larson, 1979: Fig. 2) indicates very constant rates of molecular change along most of the lines leading to the 26 living species of *Plethodon*, a result not inconsistent with the molecular clock hypothesis of reasonably constant rates of molecular evolution.

On the other hand, the cophenetic correlation coefficients for the UPGMA phenograms (calculated for the 115 most different populations, see below) of this study, 0.84 to 0.89 (Table 2), as well as those obtained in Highton's (1984) study of several *P. glutinosus* complex populations, 0.89, are substantially lower than those of the above mentioned studies. Whether this low correlation is due to the fact that there are no highly divergent outgroups included in the studies of the *P. glutinosus* complex, or to other factors (such as hybridization between adjacent populations of closely related groups or unequal rates of molecular evolution), is unknown.

Relationships Among Groups

The BIOSYS-1 program (Swofford and Selander, 1981) has made it possible to calculate several different measures of genetic distance and similarity in addition to the commonly used measures of Nei (1972, 1978). These measures include those of Rogers (1972) modified by Wright (1978), Edwards (1971; 1974), Prevosti (Wright, 1978), and two (arc distance and chord distance) of Cavalli-Sforza and Edwards (1967). Different clustering and tree-building algorithms are also available for use with various measures of genetic distance. Two were used: the phenetic UPGMA method (Sneath and Sokal, 1973) and the distance Wagner method of Farris (1972). The trees obtained by the latter method were rooted in two ways: (1) at the midpoint of the longest patristic distance, and (2) using the most divergent group (*P. kentucki*) as an outgroup.

Unfortunately, it was not possible to use the BIOSYS-1 program for all 135 samples because of a lack of computer capacity. By removing 20 samples, however, the program could be used for the remaining 115 populations. I chose to remove the 20 populations that are closest genetically to other samples in the study. Thus no population sample was removed that had a D of more than 0.005 to any other sample, so practically all of the geographic genetic variation present in the original 135 populations still remains. Samples removed were 4, 13–15, 17, 18, 56, 58, 60, 62, 63, 65, 90, 95, 106–110, and 113. The UPGMA phenogram of the I values of the 115 selected samples was compared to the complete phenogram with all 135 populations; the two are virtually identical except for a few minor changes in branching order for the most similar populations of Group 6.

Table 2. Goodness-of-fit statistics for 115 samples of the *P. glutinosus* complex.

	UPGMA Phenograms								
	Nei (1972)		Rogers (1972)		Wright (1978)	Prevosti see Wright	Cavalli-Sforza and Edwards (1967)		Edwards (1971; 1974)
	I	*D*	*D*	*S*	modification	(1978)	Chord	Arc	
Farris *F*	246.7	325.7	229.3	229.3	263.3	241.9	222.4	259.1	355.8
Prager and Wilson *F*	4.8	19.2	4.7	13.8	9.1	13.7	8.0	8.6	8.7
Fitch and Margoliash % S.D.	6.6	36.6	6.3	22.1	13.9	22.1	12.1	13.1	12.8
Cophenetic *r*	0.85	0.84	0.86	0.86	0.87	0.86	0.89	0.88	0.87
	Distance Wagner Trees (after optimization)								
Farris *F*					300.7	255.8	246.2	263.7	341.8
Prager and Wilson *F*					10.4	14.5	8.9	8.7	8.4
Fitch and Margoliash % S.D.					14.5	20.9	12.8	12.4	11.8
Cophenetic *r*					0.88	0.87	0.90	0.89	0.91

The UPGMA phenograms of all the different genetic similarity and distance measures are similar with only minor differences in branching order of closely related populations. In all the phenograms, Group 16 is the most different and groups 1, 14, and 15 almost always separate from all other groups near the base of the phenogram. Groups 2, 3, and 4 always cluster together as do groups 5 and 6, groups 9 and 10, and groups 11 and 12 (with one exception: on the Edward's distance phenogram, Group 12 clusters with Group 1 rather than with Group 11; it probably belongs with Group 1 [see below]). Groups 7 and 8 do not always cluster together but are sometimes associated with several other different groups before they group together.

Although all the UPGMA phenograms using the different measures of genetic distance are similar to one another, the distance Wagner trees often show major differences in the relationships of groups. For example, Group 16 (*P. kentucki*) is the most divergent group on all 11 phenograms, but on the five trees rooted at the midpoint of the largest patristic distance, Group 16 clusters most closely with Group 14 on four trees and with Group 15 on one tree. *P. kentucki* and its sister group cluster most closely to groups 2, 3, and 4 on two trees, Group 5 on one tree, Group 12 on one tree, and Group 13 on one tree.

P. aureolus (Group 15) and *P. teyahalee* (Group 1) are either the second or third most different groups in 10 of 11 UPGMA phenograms. However, on the distance Wagner trees rooted at the midpoint of the largest patristic distance, *P. aureolus* clusters most closely to groups 1 and 12 on one tree, Group 7 on the second, Group 16 on the third, groups 1, 12, and 13 on the fourth, and groups 8, 9, and 10 on the fifth tree! On the other hand, the relationship of *P. teyahalee* is the same on all the trees; it is the sister group of population 115 of Group 12. This relationship is significant in understanding the genetic relationships of groups 1 and 12 as discussed below. Because of the many differences among the various trees and phenograms, it is not possible to suggest a phylogeny of the groups. Although *P. kentucki* and *P. aureolus* are the most differentiated forms and either one or both probably differentiated from the proto-*glutinosus* stock earlier than the other groups, so many different trees might be expected if most of the remaining groups differentiated from one another at approximately the same time. Under this hypothesis, groups 1 to 14 might have begun diverging from each other at a time when climatic conditions were conducive to the subdivision of the species into many geographic isolates. This pattern

of subdivision exists in the closely related species, *P. jordani*, whose range is now subdivided into 22 geographic isolates (Highton, 1970, 1972). That some groups are slightly more closely related to others might be partly the result of hybridization and exchange of genes between parapatric groups and partly due to chance (in the case of allopatric forms).

Although some parapatric groups are apparently more closely related than most other groups (e.g., groups 2–4, groups 5 and 6, and groups 1 and 12), probably most have been separated for a long time, perhaps 2–6 million years, based on the molecular clock estimates of Nei distances (Maxson and Maxson, 1979). Some of the groups may have had smaller ranges then they do now and might have been isolated from one another by the cold and dry climates of the Pliocene and Pleistocene (Davis, 1983; Delcourt, 1979; Whitehead, 1981; Wolfe, 1978), but it is not clear how all of the forms might have been isolated. Some groups may never have received gene flow from adjacent groups when separated by such major barriers as the Mississippi River (between groups 6 and 13, 8 and 9, and 8 and 13). The present distribution of the groups does not favor the isolation-by-distance model of differentiation (Wright, 1943, 1969) because there are usually abrupt changes in allele frequencies at the boundaries of groups rather than gradual clinal increases in genetic distance with geographic distance. Moreover, geographically distant groups are not consistently more different genetically from those near by.

Thus I hypothesize that *P. kentucki* diverged first, then *P. aureolus*, then slightly later all or most of the other 14 groups were isolated at approximately the same time. Subsequently, there probably has been occasional interbreeding between some of the parapatric groups and some influence of this hybridization is still present in the descendants of the affected populations. Because of the large amount of genetic divergence that has already taken place, the effects of gene flow have been minimal in the recent past and will probably be even less in the future. This trend would probably have been true even without the human disturbance of habitats, but the destruction of so many of the woodlands to which these salamanders are adapted will make gene flow between adjacent groups even less frequent in the future. The *I* and *D* values of the comparisons of all the groups are shown in Table 3.

Table 2 shows four goodness-of-fit statistics for the different phenograms and trees: Farris' (1972) "*F*," Prager and Wilson's (1976) "*F*," Fitch and Margoliash's (1967) "percent standard deviation," and Sokal and

Table 3. Within-group *I*, range, standard deviation of *I*, and *N* (on diagonal), between-group statistics (below diagonal), and between-group *D* (above diagonal).

GROUP (Population)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 (1–6)	0.99 0.98–1.0 0.005 15	0.39	0.41	0.42	0.42	0.38	0.39	0.35	0.24	0.38	0.28	0.20	0.35	0.40	0.42	0.45
2 (7–10)	0.68 0.64–0.75 0.03 24	0.91 0.88–0.95 0.02 6	0.21	0.25	0.36	0.26	0.39	0.30	0.42	0.40	0.31	0.37	0.29	0.34	0.39	0.51
3 (11–22)	0.66 0.59–0.75 0.03 72	0.81 0.73–0.87 0.04 48	0.93 0.83–1.0 0.06 66	0.20	0.37	0.27	0.30	0.25	0.37	0.40	0.26	0.30	0.33	0.36	0.44	0.50
4 (23–29)	0.66 0.59–0.74 0.04 42	0.78 0.69–0.84 0.04 28	0.82 0.75–0.90 0.03 84	0.91 0.85–0.99 0.04 21	0.36	0.26	0.36	0.30	0.38	0.33	0.30	0.30	0.36	0.23	0.46	0.48

GROUP (Population)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
5	0.66	0.70	0.69	0.69	0.93	0.18	0.35	0.27	0.40	0.39	0.22	0.29	0.33	0.25	0.39	0.56
(30–34)	0.63–0.69	0.64–0.77	0.65–0.73	0.60–0.83	0.86–0.99											
	0.02	0.04	0.02	0.06	0.04											
	30	20	60	35	10											
6	0.68	0.77	0.76	0.77	0.83	0.95	0.28	0.19	0.34	0.27	0.20	0.28	0.24	0.28	0.31	0.49
(35–74)	0.61–0.77	0.68–0.86	0.64–0.83	0.63–0.92	0.74–0.93	0.82–1.0										
	0.03	0.04	0.04	0.07	0.03	0.04										
	240	160	480	280	200	780										
7	0.68	0.68	0.74	0.70	0.70	0.76	0.97	0.17	0.24	0.23	0.18	0.23	0.25	0.35	0.29	0.49
(75–76)	0.63–0.72	0.62–0.71	0.67–0.77	0.66–0.76	0.65–0.73	0.70–0.80	—									
	0.03	0.03	0.03	0.03	0.02	0.02	—									
	12	8	24	14	10	80	1									
8	0.71	0.74	0.78	0.74	0.76	0.83	0.85	0.94	0.18	0.20	0.17	0.24	0.29	0.28	0.24	0.47
(77–87)	0.66–0.76	0.69–0.79	0.64–0.84	0.64–0.88	0.69–0.81	0.68–0.94	0.78–0.89	0.85–1.0								
	0.02	0.02	0.05	0.06	0.03	0.04	0.03	0.04								
	66	44	132	77	55	440	22	55								

9	0.79	0.65	0.69	0.68	0.67	0.71	0.79	0.83	0.97	0.16	0.25	0.20	0.32	0.38	0.35	0.50
(88–90)	0.74–0.82	0.62–0.72	0.63–0.76	0.62–0.77	0.66–0.71	0.64–0.80	0.76–0.82	0.78–0.89	0.95–1.0							
	0.03	0.03	0.03	0.04	0.02	0.04	0.03	0.03	0.03							
	18	12	36	21	15	120	6	33	3							
10	0.69	0.67	0.67	0.72	0.68	0.76	0.80	0.82	0.86	—	0.31	0.26	0.29	0.37	0.38	0.56
(91)	0.66–0.71	0.66–0.69	0.66–0.69	0.67–0.80	0.63–0.70	0.68–0.81	0.79–0.80	0.76–0.90	0.84–0.89							
	0.02	0.01	0.01	0.04	0.03	0.02	0.01	0.05	0.03							
	6	4	12	7	5	40	2	11	3							
11	0.76	0.74	0.77	0.74	0.80	0.82	0.83	0.84	0.78	0.73	0.93	0.15	0.19	0.24	0.27	0.40
(93–102)	0.69–0.80	0.67–0.80	0.68–0.84	0.67–0.85	0.72–0.91	0.72–0.94	0.78–0.89	0.75–0.91	0.72–0.83	0.71–0.76	0.83–1.0					
	0.02	0.03	0.04	0.05	0.04	0.04	0.03	0.03	0.03	0.02	0.04					
	60	40	120	70	50	400	20	110	30	10	45					
12	0.82	0.69	0.74	0.74	0.75	0.76	0.79	0.79	0.82	0.77	0.86	0.93	0.27	0.29	0.37	0.43
(103–115)	0.75–0.93	0.59–0.78	0.63–0.85	0.66–0.87	0.71–0.80	0.67–0.87	0.73–0.85	0.69–0.88	0.78–0.88	0.73–0.84	0.78–0.93	0.83–1.0				
	0.04	0.05	0.04	0.04	0.02	0.04	0.03	0.04	0.02	0.03	0.03	0.05				
	78	52	156	91	65	520	26	143	39	13	130	78				
13	0.70	0.75	0.72	0.70	0.72	0.78	0.78	0.75	0.72	0.75	0.83	0.76	0.90	0.32	0.33	0.42
(92,116–127)	0.65–0.82	0.67–0.79	0.67–0.81	0.61–0.87	0.64–0.83	0.68–0.90	0.71–0.88	0.62–0.87	0.67–0.84	0.69–0.83	0.73–0.93	0.70–0.89	0.78–0.99			
	0.04	0.02	0.03	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.06			
	78	52	156	91	65	520	26	143	39	13	130	169	78			

GROUP (Population)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
14	0.67	0.71	0.70	0.79	0.78	0.75	0.71	0.75	0.68	0.69	0.79	0.75	0.73	—	0.42	0.43
(128)	0.65–0.69	0.66–0.74	0.68–0.73	0.77–0.81	0.77–0.82	0.72–0.84	0.69–0.73	0.72–0.80	0.68	—	0.75–0.82	0.72–0.78	0.67–0.82			
	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.03	0	—	0.02	0.02	0.05			
	6	4	12	7	5	40	2	11	3	1	10	13	13			
15	0.65	0.67	0.64	0.63	0.68	0.73	0.75	0.79	0.70	0.68	0.76	0.69	0.72	0.66	0.89	0.51
(129–131)	0.58–0.73	0.62–0.72	0.57–0.71	0.57–0.73	0.62–0.74	0.64–0.81	0.70–0.80	0.74–0.82	0.66–0.72	0.65–0.73	0.64–0.86	0.58–0.80	0.62–0.80	0.63–0.68	0.87–0.92	
	0.05	0.03	0.04	0.01	0.03	0.03	0.04	0.02	0.02	0.04	0.07	0.06	0.05	0.03	0.03	
	18	12	36	21	15	120	6	33	9	3	30	39	39	3	3	
16	0.64	0.60	0.61	0.62	0.57	0.61	0.61	0.63	0.60	0.57	0.67	0.65	0.66	0.65	0.60	0.90
(132–135)	0.60–0.66	0.55–0.67	0.55–0.67	0.57–0.68	0.55–0.60	0.54–0.67	0.59–0.63	0.58–0.66	0.60–0.62	0.56–0.58	0.61–0.71	0.60–0.69	0.62–0.71	0.62–0.68	0.56–0.64	0.86–0.96
	0.02	0.03	0.02	0.03	0.01	0.03	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.03	0.03	0.03
	24	16	48	28	20	160	8	44	12	4	40	52	52	4	12	6

Rohlf's (1962) cophenetic correlation coefficient (r_{CS}). The lowest values of the first three coefficients and the highest r_{CS} should be seen when the trees best fit the distance data. The use of Fitch and Margoliash (1967) trees was not considered because Farris *et al.* (1982) have found that they are less efficient than distance Wagner trees, and most workers have not been able to find the best tree from the large number generated. Fitch-Margoliash trees would be even more of a problem with the *P. glutinosus* data set because of the large number of populations.

The BIOSYS-1 program (Swofford, 1981) also provides optimized distance Wagner trees to make the goodness-of-fit statistics comparable to those of the other clustering methods. All of these trees show much less variance in branch lengths than the unoptimized distance Wagner trees. Two of the unoptimized distance Wagner trees with the best goodness-of-fit statistics (see Table 2) are shown in Figure 6. The goodness-of-fit statistics are not very different from those of the UPGMA phenograms.

All the populations of Group 1 (*P. teyahalee*) are closely associated on all of the distance Wagner trees, but instead of branching off early as an independent line as they do on the UPGMA phenograms, on all the trees Group 1 clusters with population 115 of Group 12. This arrangement probably indicates the evolutionary relationships of these two groups rather than the relationship indicated by the UPGMA phenograms. Groups 1 and 12 are the only large-sized, white-spotted forms and their ranges are parapatric, separated by the French Broad River. This river also appears to limit the ranges of *P. cinereus* and *P. serratus* (Highton and Webster, 1976). Population 115 is from the mountains near the headwaters of this river and it is the member of Group 12 that is closest genetically to Group 1. Group 1 has a lower average D value (0.20) to Group 12 than to any other group (Table 3). A comparison of *P. teyahalee* with other southern Appalachian large *Plethodon* by Peabody (1978) shows it to be more closely related to *P. jordani* populations of the Nantahala and Unicoi mountains (Table 4, group D) than it is to any of the southern Appalachian types of the *P. glutinosus* complex. *P. teyahalee* has common alleles at two loci that are unique (Pgi *c* and Trf *a*), two that are fixed for alleles that are common in Nantahala Mountain *P. jordani* (Alb-*c* and Pt-2 *b*), and two that are common in Group 12 (Est-*e* and Got-1 *h*).

Salamanders of Group 1 often possess small red spots on their legs, a character much more abundant in Nantahala Mountain *P. jordani* (Highton, 1962b). Both the genetic data and the morphological data

Fig. 6. Distance Wagner trees, rooted by using Group 16 (*P. kentucki*) as an outgroup, calculated from Edward's distances and Cavalli-Sforza and Edwards' chord distances.

suggest, therefore, that *P. teyahalee* might have had a hybrid origin as a result of interbreeding between Group 12, white-spotted *P. glutinosus*, and red-legged Nantahala Mountain *P. jordani*. At the present time, *P. teyahalee* hybridizes extensively with Nantahala and Unicoi Mountain *P. jordani* (Highton, 1970) at all known contacts of their ranges, although it rarely hybridizes with the other geographic populations of *P. jordani* with which it is widely sympatric. The hypothesis of a hybrid origin of *P. teyahalee* thus is consistent with the genetic data, and its close genetic relationship to *P. jordani* may explain why it hybridizes frequently only with the Nantahala and Unicoi mountain populations of that species. Because of its hybrid origin, *P. teyahalee* might be expected not only to share far more genes with these populations of *P. jordani*, but the two might also have fewer reproductive isolating mechanisms. Judging from the small amount of geographic genetic variation in *P. teyahalee*, its origin by hybridization may have been a rather recent event, or it may have expanded its range only recently.

The length of the branch leading to *P. teyahalee* on all distance Wagner trees indicates more molecular evolution on that line than on the branches leading to populations of Group 12. This result would be expected if there had been an infusion of foreign (*P. jordani*) genes into the ancestor of *P. teyahalee*. By this hypothesis of the origin of *P. teyahalee*, the difference in the apparent rates of molecular evolution as observed in the distance Wagner trees would be more the result of hybridization than of differential substitution rates. By assuming equal rates of evolution on all lines and by averaging *I* values between groups, the UPGMA phenogram fails to indicate this evolutionary interaction. On the other hand, some of the distance Wagner trees indicate close relationship between groups based on similarities between single populations of each group (perhaps due to gene flow) or similarities due to slower than average rates of molecular change rather than real genetic relationships (see below).

The four populations of Group 2 cluster together on all distance Wagner trees just as they do on the phenograms. On the distance Wagner trees they are indicated as closer to Group 3 than to Group 4, whereas on the phenograms groups 3 and 4 are sister groups. The mean *D* is 0.21 between groups 2 and 3, 0.25 between groups 2 and 4, and 0.20 between groups 3 and 4. If there is some gene flow between the parapatric groups, then it might be expected that the two allopatric groups (2 and 4) might be slightly more different genetically. The three

groups are clearly more closely related to each other than they are to any other group.

The southern populations of Group 3 have a considerable amount of geographic variation. As noted above, the seven northernmost populations (13–19) of this group are almost identical genetically. These same populations have very low variability. If this region was recently reoccupied by salamanders from the coastal plain of southern North Carolina (the retreat of the Wisconsin glaciation began only about 15,000 years ago), then they would be expected to be genetically similar, particularly because of their low variability. The most differentiated populations of Group 3 are those from the northwestern part of its range (20–22).

The seven South Carolina and Georgia coastal plain populations of Group 4 (23–29) show a considerable amount of local genetic differentiation. The most different populations are the two from Georgia (28 and 29) from just west of the Savannah River. One of these (29) is so different that it clusters with other groups on 3 of 10 of the distance Wagner trees. All individuals of the two samples from the west side of the Savannah River are spotted dorsally and laterally, but all the known populations from just east of the river in Jasper County, South Carolina, are unspotted.

The five populations of Group 5 always cluster together. They are closest genetically to Group 6 ($\bar{D} = 0.18$) but are more different from other parapatric populations of groups 4 ($\bar{D} = 0.36$), 11 ($\bar{D} = 0.22$), and 14 ($D = 0.25$).

Group 6 is the largest group and consists of 40 samples (35–74). Genetically, the northern 32 populations (39–71) are closely related and 24 populations (47–70) are very similar. The latter group probably spread northward only recently after the retreat of the Wisconsin glaciation made the area again habitable. Even now, after thousands of years of warming of the region, it has barely been able to enter glaciated territory in the extreme northern part of its range. This limited movement is in sharp contrast to the great distance that its congener, *P. cinereus*, must have dispersed northward (Grobman, 1944). *P. glutinosus* is more active during warm weather than sympatric *P. cinereus* (Highton, 1972), and the latter species ranges only as far south as North Carolina, compared to the extensive southern distribution of the *P. glutinosus* complex as far as the Gulf coast. Although none of the peripheral samples show any evidence of gene flow with Group 13 (groups 6 and 13

appear to be completely isolated by the Mississippi River), there is some genetic evidence of gene flow at the periphery of the contacts of Group 6 with other parapatric groups. Sample 71 shows decided genetic influence of Group 8 and indeed clusters with that group on all but one of the distance Wagner trees. Samples 72 and 73 show some genetic influence of Group 5 and cluster most closely with the latter on 5 of the 10 distance Wagner trees. Sample 97 (of Group 11) from the Coastal Plain physiographic province of southern Alabama shows considerable genetic influence of Group 6. Indeed, sample 97 is so close to Group 6 that only a small change in its mean D value to the latter group would place it with that group in the UPGMA phenogram of Nei's I values. Group 6 also hybridizes with Group 12 (Highton, 1972, and unpublished data) and with Group 1 (unpublished data) where their ranges contact, although groups 1 and 6 have apparently evolved reproductive isolation at one site in Polk County, Tennessee (Highton, 1984).

Group 7 includes only two samples (75 and 76) from the Ouachita Mountains of Oklahoma. It is different from other nearby populations as noted by Duncan and Highton (1979). Genetically, Group 7 is closer to groups 8 ($\bar{D} = 0.17$) and 11 ($\bar{D} = 0.18$), east of the Mississippi River, than it is to parapatric western populations of groups 9 ($\bar{D} = 0.24$), 10 ($\bar{D} = 0.23$), and 13 ($\bar{D} = 0.25$) (Table 3). However, on 6 of the 10 Wagner trees it clusters with some of the above western groups. It is not clear why the populations from Kiamichi (sample 75) and Round (sample 76) mountains (all part of the same continuous ridge) from southeastern Oklahoma are so highly differentiated (Group 10 is also quite distinct) when Group 13 is so widely distributed from Missouri to Texas. There must have been considerable isolation of populations in the Ouachita Mountain region since the Pliocene, as also indicated by the even greater amount of differentiation of the salamanders allied to *P. ouachitae* in that area (Duncan and Highton, 1979).

Group 8 includes 11 samples (77–87) from the western Gulf Coastal Plain physiographic province of western Alabama, Mississippi, southeastern Louisiana, and western Tennessee, and all are rather closely associated on both the UPGMA phenograms and all the distance Wagner trees. It also occurs in western Kentucky. Its closest relatives on most of the distance Wagner trees are groups 9 and 10, but on the UPGMA phenograms Group 7 is closest.

Group 9 includes three samples (88–90) from the north-central Louisiana and southern Arkansas Coastal Plain physiographic prov-

ince. On all distance Wagner trees and phenograms this group is closest to Group 10 (population 91). Groups 9 and 10 in turn are closest to Group 8 on all the distance Wagner trees and the UPGMA phenograms.

Group 11 consists of 11 populations (samples 93–102) from the southeastern Coastal Plain physiographic province of Florida, southern Georgia, and southern Alabama. The UPGMA phenograms show it to be closest to Group 12 ($\bar{D} = 0.15$), but it is also close to Group 8 ($\bar{D} = 0.17$), Group 7 ($\bar{D} = 0.18$), Group 13 ($\bar{D} = 0.19$), and Group 6 ($\bar{D} = 0.20$). In appearance the salamanders of this group are more similar to groups 6, 7, 8, and 13 than to Group 12. Indeed, the large-sized, white-spotted members of Group 12 are among the most different animals in appearance to Group 11. An examination of the distance Wagner trees, however, sheds more light on their relationships. On some of the trees (e.g., the Edwards distance Wagner tree in Fig. 6), the populations of Group 11 are all located near the base of the tree. This location suggests a slower rate of molecular evolution in this group than in the other groups. Thus, if through sampling errors or slower rates of change in these 22 loci, Group 11 has changed less than the others, it would be expected to cluster on the UPGMA phenograms with the group(s) that had also changed less from the common ancestor, in this case Group 12 and population 92 of Group 13. Its closest relatives are probably the adjacent members of the parapatric groups 5, 6, and 8.

The relationships of the 13 populations (103–115) of Group 12 have been discussed under groups 1 and 11. As in the northern populations of groups 3 and 6, the six northern samples of this group (105–110) are also genetically similar to each other. The remaining, more southern, populations have a considerable amount of local genetic differentiation.

The 13 populations of Group 13 (92, 116–127) are closer genetically to those of Group 11 ($\bar{D} = 0.19$) than to any other group. This finding is surprising in light of the geographic proximity of groups 7, 9, and 10 ($\bar{D} = 0.25$, 0.32, and 0.29, respectively) but is probably explained by the apparently slow rate of molecular evolution in Group 11.

Group 14, a single highly differentiated population (128), is closest genetically to the geographically nearby groups 4, 11, and 5 ($\bar{D} = 0.23$, 0.24, and 0.25, respectively). Two of the distance Wagner trees place this group closest to Group 5, four put it closest to Group 16, and in the remaining four it is found near the base of the line leading to all groups other than Group 16. The close relationship of groups 14 and 16, indicated by distance Wagner trees when they are genetically so differ-

ent ($\bar{D}$ = 0.43), indicates a problem with some of the distance measures, the distance Wagner method, or both, for use in phylogenetic reconstruction of electrophoretic data.

The three populations of Group 15 (*P. aureolus*) are very different from all others. Although they are closest genetically to Group 8 ($\bar{D}$ = 0.24), they are very different from these populations. In the various distance Wagner trees Group 15 clusters with groups 1, 7, 8, 9, 10, 12, 13, and 16! Considering the high amount of genetic distance that has been built up in *P. aureolus* from all other groups, it is surprising that in only 1 of 10 trees it appears as an outgroup to all the other groups except *P. kentucki* (the Edwards distance Wagner tree in Fig. 6). The latter arrangement is probably a better indication of its phylogenetic relationships.

Group 16 (*P. kentucki*) is by far the most different group on the UPGMA phenograms. As in the case of *P. aureolus*, it clusters with several different groups on the various distance Wagner trees rooted at the midpoint of the greatest patristic distance.

Pleistocene Effects on Interpopulation Relationships

Highton and Webster (1976) found that all samples of *Plethodon cinereus* from the northern three-quarters of its range (those occupying glaciated territory) were almost identical genetically. This genetic similarity may indicate that all were derived from the most northern, closely related populations that survived the Wisconsin glaciation and were in the most favored position to disperse northward upon its retreat. Larson (1984) and Larson *et al.* (1984) have discussed gene flow in these populations of *P. cinereus*. Since the Wisconsin glaciers began their withdrawal only about 10,000–15,000 years ago, probably little molecular evolution would have occurred in northern populations and they all might still be very similar to their common ancestor. In the *P. glutinosus* complex, genetic uniformity is also apparent in the most northern populations within several of the groups. Davis (1983), Delcourt (1979), and Whitehead (1981) review changes in the eastern forest types during the last 30,000 years.

After the last Pleistocene glacial maximum, Group 3, whose northernmost range probably was limited to the Coastal Plain physiographic province of South Carolina or southern North Carolina, probably moved northward in the coastal plain to southern Virginia. It apparently was able to cross the James and York rivers, but this group is not

known north of the Rappahannock River or on the Del-Mar-Va Peninsula, across Chesapeake Bay, where the habitat appears ideal for this form that is adapted to the coastal plain.

Group 12 probably moved northward from northern North Carolina or southern Virginia in the Piedmont, Blue Ridge, and the eastern Valley and Ridge physiographic provinces until it reached the Potomac River, which it apparently was unable to cross except in Washington County, Maryland (Highton, 1972). It has also extended its range into the coastal plain of Virginia in the "northern neck" (between the Potomac and Rappahannock rivers), as well as the area just south of the Rappahannock River.

Group 6 probably moved northward from the Appalachian Plateau of southern Kentucky and spread to Ohio, Pennsylvania, Maryland, New Jersey, and New York. Because Group 12 was unable to cross the Potomac River (except in Washington County, Maryland), Group 6 was able to migrate eastward into the Blue Ridge and Piedmont provinces of Maryland and Pennsylvania, provinces that are occupied solely by Group 12 south of the Potomac River. Apparently Group 6, unlike Group 12, is not adapted to life in the coastal plain, for it appears to be absent from that province in Long Island, New Jersey, Delaware, and Maryland (Highton, 1962b, 1972).

Populations of Group 6 in Illinois (41 and 42) and western Indiana (40 and 44) are genetically closest to those in western Kentucky (39 and 43) and southeastern Tennessee (45). It appears that these western populations of Group 6 crossed the Ohio River (in one direction or the other) during the middle or late Pleistocene, subsequent to the genetic differentiation of some of the major subgroups of Group 6.

Northern populations of Group 13 are not as similar genetically to each other as are those of the other three northern groups (3, 6, and 12), but the three Missouri samples (118–120) and the northernmost Arkansas sample (121) are among the most closely related members of that group. The latter four populations are also very similar to the dark-throated Texas populations (122 and 123). Whether this close relationship is the result of recent colonization of Texas by populations from the northern part of the range of Group 13, or colonization in the opposite direction at the end of the last glaciation by populations from Texas, cannot now be determined.

The only other groups containing closely related populations are groups 1, 8, and 9. Group 1, as suggested above, may be of hybrid origin

and this event may have taken place rather recently, thus leaving little time for subsequent local differentiation. The populations of Group 8 that are all very closely related (82–86, from Mississippi and southeastern Louisiana) are unusual in that most other southeastern coastal plain populations of groups 4, 5, and 11 have a considerable amount of within-group local differentiation. If the rise in sea levels during interglacial periods of the Pleistocene were responsible for exterminating populations in low-lying areas, then those populations that result from recolonizations might show less local differentiation than those from adjacent higher areas. However, it is not clear why this pattern is seen in Mississippi but not in Alabama, Florida, Georgia, and South Carolina. Perhaps the southern populations of Group 8 only recently colonized that area because it was previously uninhabitable for some unknown reason.

Analysis of Substitutions within Loci

Variation in rates of substitutions at the structural gene loci analyzed in this study is shown in Table 4. Of the 22 loci, 4 (α-Gpd, Ldh(h), Mdh-1, and Pt-3) have the same common (usually fixed) allele in all groups. Because the same alleles are also common or fixed in the other species of the *P. glutinosus* group, these loci are presumably changing at very slow rates in these salamanders. Five loci (Alb, Est, Got-1, 6-Pgd, and Trf) appear to be evolving at very rapid rates, in agreement with the conclusion of Sarich (1977) that plasma proteins and esterases are among the most rapidly evolving loci in vertebrates. The remaining 13 loci appear to be evolving at intermediate rates and are useful in attempting to determine the ancestral allele when compared to the six species most closely related to *P. glutinosus* and the other two species of eastern large *Plethodon* (*P. wehrlei* and *P. punctatus*) of the *P. wehrlei* group used as an outgroup. One or more substitutions have occurred in some of the intermediate or fast evolving loci in all 16 groups, as seen in Table 4. The data on the five related species were obtained from comparisons of *P. glutinosus* alleles with the samples of *P. jordani* used by Peabody (1978), and for other species by Duncan and Highton (1979), and Wynn, Highton and Jacobs (1988, and unpublished data). Highton and MacGregor (1983) and Duncan and Highton (1979) found that *P. yonahlossee* is genetically the most different member of the species group. The other five species (*P. jordani*, *P. fourchensis*, *P. ouachitae*, *P. caddoensis*,

Table 4. The most common allele in the species of eastern large *Plethodon*; *f* = *P. fourchensis*; *o* = *P. ouachitae*; *c* = *P. caddoensis*; *pe* = *P. petraeus*; *y* = *P. yonahlossee*; *w* = *P. wehrlei*; *pu* = *P. punctatus*.

	P. glutinosus group																														
	P. glutinosus complex group																*P. jordani* complex group (Peabody 1978)										*P. wehrlei* group *(outgroup)*		Probable ancestral allele of *P. glutinosus* species group
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E	f	o	c	pe	y	w	pu	
Fast-evolving loci																															
Alb	c	d	d	d	h	d	e	d	c	d	e	e	e	h	e	a	e	e	b^+	c	f	a	b	a^+	d	g	a^+	a^{++}		?
Est	e	p	t	p	k	t	t	t	l	p	t	e	t	p	t	t	y	t	t	d	aa	y	p	z	r	q	f	p		?
Got-1	h	j	j	j	j	j	b	j	h	h	j	h	j	j	j	d	h	h	j	j	j	f	b	a	a	g	h^-	f		?
6-Pgd	b	d	b	c	b	d	d	b	b	d	b	b	d	c	b	c	d	b	c	b	b	b	a^-	b	b^+	f	c	c		?
Trf	a	h	o	o	i	o	i	l	k	k	l	i	k	h	f	m	g	e	j	j	h	o	o	q	k	i	h	f		?

Intermediate-evolving loci																													
Fum	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	c	b	b	b	b	b	b
Gdh	b	b	b	b	a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
Got-2	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	d	e	e	e	e	e	e	e	e	e	e	f	f	e
Idh-1	b	e	b	e	b	b	b	b	b	b	b	b	b	d	b	b	b	b	e	e	b	b	b	b	b	e	e	e	e
Idh-2	c	c	c	c	c	c	c	c	c	c	c	c	c	c	d	e	e	e	e	c	c	c	c	c	c	e	e	e	e
Ldh-(m)	h	h	h	h	g	g	h	h	h	h	h	h	h	h	f	h	h	h	h	h	h	k	h	h	j	h	h	h	h
Lap	a	a	c	b	a	a	c	a	a	a	a	a	a	a	a	a	a	a	a	a	c	c	c	c	a	a	a	a	a
Mdh-2	d	d	d	d	d	d	d	d	d	a	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d^-	d^-	d
Pep	d	c	c	d	d	d	d	d	d	d	d	d	b	d	d	d	d	d	d	d	d	d	d	d	a	g	a^+	a^+	d
Pgi	c	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g
Pgm	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c^+	c^+	c
Pt-1	b	b	b	b	b	b	c	c	c	c	b	b	b	b	c	b	b	b	b	b	b	b	b	b	b	b	c^+	c^+	b
Pt-2	b	c	a	a	a	a	a	a	a	a	a	a	h	a	d	g	e	b	b	b	e	e	e	e	e	e	e	e	e
No. Subs.*	4	3	5	3	5	4	5	4	4	5	3	3	4	3	5	3	1	2	1	2	3	4	4	3	4	1			

*Probable number of substitutions in intermediate-evolving loci from common ancestor of *P. glutinosus* Group.

and *P. petraeus*) also differ from all of the groups of the *P. glutinosus* complex.

Which alleles of the five fast-evolving loci were present in the common ancestor of the group cannot be determined because of the large amount of variation now present. On the other hand, for the remaining 13 variable loci apparently evolving at slower rates, the likely ancestral allele can be selected for the 16 groups of the *P. glutinosus* complex with the aid of the data on the related species used as outgroups (Table 4). For example, at the Gdh locus, allele *b* is very common or fixed in all forms with the exception of *P. glutinosus* complex Group 5. Thus it is very likely that the substitution of allele *a* occurred during the differentiation of Group 5, although two of the five samples of this group (31 and 32, see Appendix 2) still have a low frequency of allele *b*. It is similarly possible to suggest the probable ancestral allele at some of the other loci evolving at intermediate rates. In the case of Pt-2, the probable ancestral allele (*e*) is not present in any of the 135 samples of the *P. glutinosus* complex. However, because allele *e* is not only present in the two outgroup species, but in seven forms of the *P. glutinosus* species group, it is likely that it is the ancestral type.

For each *P. glutinosus* group species, the number of loci evolving at intermediate rates that have probably had electrophoretically detectable substitutions replace (or become commoner than) the ancestral allele is shown on the bottom line of Table 4. Since the time that all species had a common ancestor, the number of loci evolving at intermediate rates varies from one to five with groups 3, 5, 7, 10, and 15 having the most. Groups 11 and 12 have had fewer substitutions and are the same at all intermediate-evolving loci, and it is therefore not surprising that they have such a low $\bar{D}$ value because the contribution to D between groups 11 and 12 is almost entirely due to the few fast-evolving loci. From the present data it is not possible to determine whether their similarity is because of close evolutionary relationship, a significantly slower rate of biochemical differentiation, or simply because the loci selected for analysis by chance happened to have had fewer substitutions. The first of these possibilities appears unlikely because of the large amount of morphological divergence between the two groups and their geographic separation, but the correct choice between the second and third possibilities probably could be made by further study of additional independent genetic loci. I think that the last explanation is probably correct. Attempts to do a cladistic analysis of substitutions, such

as attempted by Wake *et al.* (1978) and Patton and Avise (1983), have proven impossible because of considerable polymorphism at some loci.

An attempt to find the relationships of the 26 forms of the *P. glutinosus* species group was made by comparing one sample of each form (usually the sample selected was from at or near the type locality) with the two species of the *P. wehrlei* group. UPGMA phenograms and distance Wagner trees all indicate that the 16 species of the *P. glutinosus* complex are not a monophyletic group, but instead cluster variously with other species of the species group. Only one pair of species of the group, *P. caddoensis* and *P. ouachitae*, cluster together consistently, as do *P. wehrlei* and *P. punctatus* of the *P. wehrlei* group. This result is interpreted to indicate that the other species also diverged from the same common ancestor at approximately the same time as the 16 members of the *P. glutinosus* complex.

Variability of Populations

The average heterozygosity of each population sample is shown in Appendix 2 and indicated geographically in Figure 7. The means and ranges for each group are shown in Table 5.

Table 5. Heterozygosity of groups.

Group	Species	Population	Mean	Range
1	*P. teyahalee*	1–6	0.05	0.02–0.09
2	*P. chattahoochee*	7–10	0.19	0.14–0.23
3	*P. chlorobryonis*	11–22	0.04	0–0.13
4	*P. variolatus*	23–29	0.07	0.04–0.10
5	*P. ocmulgee*	30–34	0.06	0.04–0.08
6	*P. glutinosus*	35–74	0.07	0.01–0.18
7	*P. kiamichi*	75–76	0.11	0.08–0.15
8	*P. mississippi*	77–87	0.06	0–0.13
9	*P. kisatchie*	88–90	0.002	0–0.004
10	*P. sequoyah*	91	0.04	—
11	*P. grobmani*	93–102	0.06	0.01–0.12
12	*P. cylindraceus*	103–115	0.05	0.02–0.10
13	*P. albagula*	92,116–127	0.06	0.01–0.14
14	*P. savannah*	128	0.0	—
15	*P. aureolus*	129–131	0.12	0.08–0.15
16	*P. kentucki*	132–135	0.13	0.11–0.15

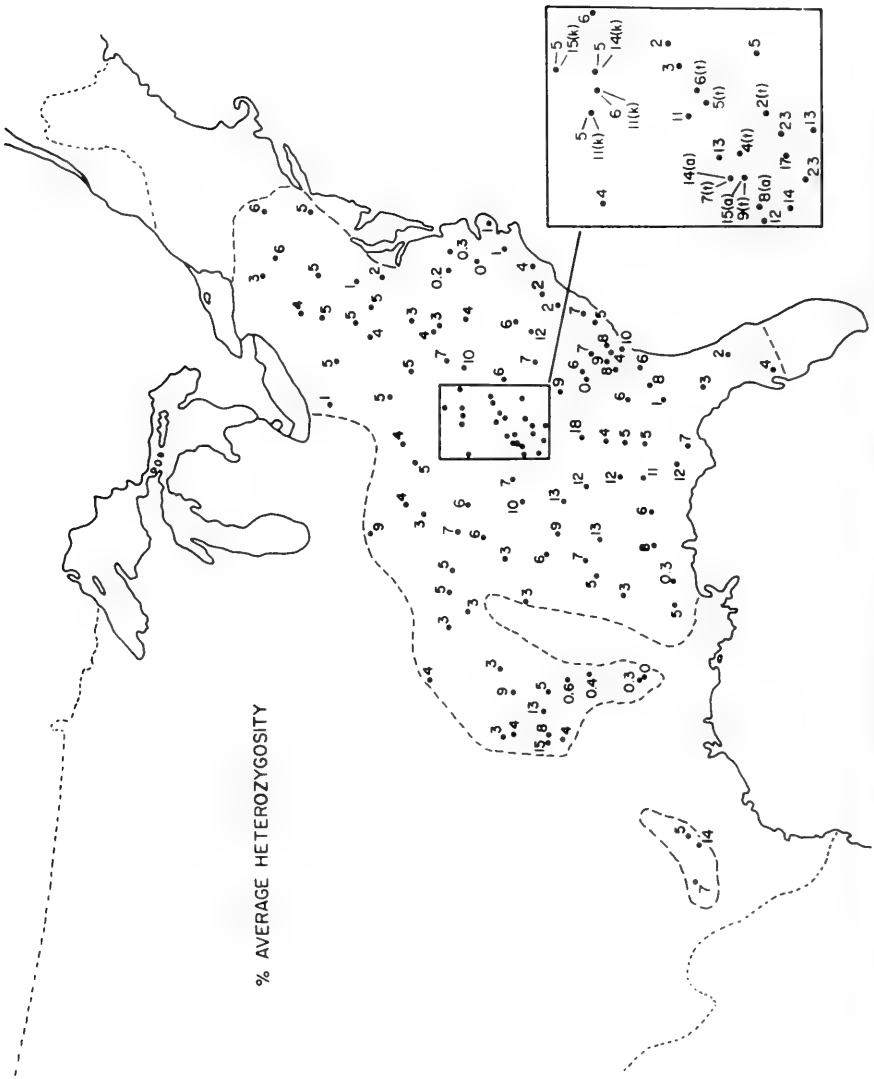

Fig. 7. Percent average heterozygosity of all samples indicated geographically. In inset, a = *P. aureolus*, k = *P. kentucki*, and t = *P. teyahalee*.

All but two of the smallest groups appear to have a considerable amount of genetic variation. Group 2 has by far the highest amount of variation with a mean $H = 0.19$ and includes the two most variable samples with $H = 0.23$. Groups 7, 15, and 16 also have a large amount of variability with mean H ranging from 0.11 to 0.13. The four samples of groups 9 and 14 have extremely low genetic variability. The mean H values of the remaining 10 groups indicate a moderate amount of genetic variability (0.04–0.07). The unweighted mean H of all 135 populations is 0.06.

With a few exceptions, there does not appear to be a consistent pattern of high or low heterozygosity at the periphery or in the center of the ranges of the various groups. This pattern agrees with that in *Drosophila* summarized in Brussard (1984). The northern populations of Group 3 all have very low heterozygosity and all are virtually identical genetically. The northern populations of groups 6, 12, and 13 have only a slightly lower than average variability. Thus only Group 3 shows the type of fixation of variable loci that might have occurred in the source populations at the northern margin of the range during the glacial maxima. Some of these populations might have become very small, living as they did at the northern limit of survivable climate and habitat where genetic drift might have resulted in the fixation of variable loci.

Because only one locus (Pt-3) is monomorphic among all samples, 95 percent of the loci studied are genetically variable.

Size Variation among Groups

Duncan and Highton (1979) mention some of the problems associated with using adult size as a taxonomic character in salamanders. In spite of the expected difficulties, they found that there is considerable uniformity in maximum size within genetically differentiated populations of the four species of the *Plethodon glutinosus* group in the Ouachita Mountains of Arkansas and Oklahoma. In the *P. glutinosus* complex, however, there appears to be an environmentally related pattern of geographic variation in maximum size. With the exception of *P. aureolus* and *P. kentucki* (small Appalachian species sympatric with larger members of the complex), size is correlated with physiographic province.

Small-sized populations (maximum size usually under 74 mm snout to anterior angle of the vent) occur in the Coastal Plain physiographic

province (Table 6). In populations in the other upland physiographic provinces, the maximum size is usually larger. These results agree with the data in Highton (1962a). In the three groups that are distributed mostly in the coastal plain but also occur in other adjacent provinces (groups 3, 5, and 8), the maximum adult size of the non-coastal plain populations is greater than that of the coastal plain populations. This pattern might be the result of ecophenotypic variation, perhaps related to nutrition. If genetically based, however, selection might favor different adult sizes in the different soil types of the two areas. The influence of the sandy soils of the coastal plain versus the rocky soils of the other provinces in relation to maximum burrow size should be investigated.

Table 6. Variation in the maximum size (snout to anterior angle of vent in life) of members of the *P. glutinosus* complex.

Group	Species	N	Range of maximum sizes (mm)	Mean
Coastal Plain populations				
3	*P. chlorobryonis*	9	59–73	68.1
4	*P. variolatus*	7	63–72	66.9
5	*P. ocmulgee*	4	64–70	65.8
6	*P. glutinosus*	1	72	72.0
8	*P. mississippi*	10	64–78	71.1
9	*P. kisatchie*	3	71–73	72.0
11	*P. grobmani*	10	63–69	66.2
13	*P. albagula*	1	70	70.0
14	*P. savannah*	1	69	69.0
Non-Coastal Plain populations				
1	*P. teyahalee*	6	72–90	81.0
2	*P. chattahoochee*	4	72–79	75.0
3	*P. chlorobryonis*	3	77–81	79.0
5	*P. ocmulgee*	1	81	81.0
6	*P. glutinosus*	39	68–89	78.7
7	*P. kiamichi*	2	70–83	76.5
8	*P. mississippi*	1	84	84.0
10	*P. sequoyah*	1	78	78.0
12	*P. cylindraceus*	13	76–88	81.4
13	*P. albagula*	12	68–82	74.5
15	*P. aureolus*	3	57–67	63.7
16	*P. kentucki*	4	64–75	68.5

Further analysis of geographic size variation will become available when my on-going study of the life histories of all eastern species of the genus *Plethodon* is completed.

Taxonomy

The mean *D* values of comparisons among all 16 groups are shown in Table 3. Of the 120 comparisons, only 14 are 0.20 or less, 36 are between 0.21 and 0.29, and 70 are 0.30 or higher. Thus there is a considerable amount of genetic divergence among most of the groups. Avise (1974) and Ayala (1975) indicate that local populations of species commonly have Nei genetic distances less than 0.1. Avise and Aquadro (1982) showed that there is considerable variation in genetic distances between congeneric species among classes of vertebrates with birds having the lowest and amphibians the highest.

Genetic distances between subspecies vary considerably because this taxonomic category is used to indicate so many different relationships. Avise (1974) states that "in many cases, biochemical systematists have not been able to distinguish subspecies which have been described by classical systematic criteria." Many of these subspecies were probably based on minor geographic morphological or color pattern differentiation and are not the result of long periods of evolutionary divergence. Some subspecies recognized by taxonomists, however, may have evolved during long periods of isolation or geographic differentiation and in these the amount of genetic divergence might be greater, as well as concordant with the geographic patterns of morphological variation. An extreme example is the designation by *Drosophila* geneticists of highly differentiated populations of the *D. willistoni* group as subspecies. These subspecies have a mean $D = 0.23$ (Ayala, 1975: Table VI). Subspecies in this group are defined by Dobzhansky *et al.* (1977: 192) as "geographically separated, showing only a trace or no ethological isolation, but producing sterile male hybrids in at least one of the reciprocal crosses" in the laboratory. Most modern taxonomists do not hold this concept of subspecies and usually recognize subspecies as differentiated, but naturally freely interbreeding, geographical representatives of a species. The mean *D* (0.23) between populations considered by Ayala (1975) to be "semispecies" (also partially reproductively isolated forms) is the same as between his "subspecies," indicating a similar amount of genetic divergence. Because his "subspecies" and

"semispecies" differ only in relative amount of reproductive isolation, as tested in the laboratory, both should be considered species taxonomically. Neither should be considered a subspecies because they have already evolved a significant degree of reproductive isolation (sterility in male hybrids).

In much of the work being done on genetic variation in vertebrate species (see Smith *et al.*, 1982, for a bibliography), evolutionary biologists do not appear willing to make the obvious taxonomic conclusions that follow from their biochemical data. For example, Larson (1983) found that one population of *Bolitoglossa occidentalis* (sample 17) was genetically much closer to another species (*B. rufescens*) than it was to the three other samples of *B. occidentalis*. Moreover, his seven samples of *B. rufescens* were obviously divided into two genetically differentiated groups. Although Larson recognized that the present taxonomic arrangement of these populations into two species (based on the presence or absence of maxillary teeth) must be in error, he did not make the taxonomic allocations of populations indicated by his genetic data, in spite of the fact that his results were based on 29–30 genetic loci and the present taxonomy is based on only one morphological character that he states "is not a reliable indicator of species boundaries." It should be pointed out, however, that the *B. rufescens* group may contain several species whose precise boundaries cannot be determined from the available data. Larson's data indicate that his populations 17–21 have been separated from populations 22–27 for approximately 12 million years. Surely these two groups (and perhaps additional subgroups within both groups) are separate species and should not continue to be taxonomically arranged in the old erroneous fashion (populations 17, 22, 26, and 27 as *B. occidentalis* and the rest as *B. rufescens*).

Why are many biologists reluctant to make the taxonomic revisions that are indicated by the genetic data obtained from their biochemical research? The reasons are probably among the following:

(1) Many biologists doing biochemical research on genetic variation are not systematists and do not understand or care to employ the procedures used by taxonomists to incorporate the conclusions of studies on the relationships of organisms into the formal system of nomenclature. Some journals do not accept species descriptions and these must therefore appear in separate papers from the biochemical results.

(2) In the absence of direct information on reproductive isolation,

some workers are hesitant to recognize taxa that are differentiated genetically but not morphologically (Wake, 1981), or in which the biochemical data disagree with the morphological data, since the present taxonomic system is based largely on morphology.

(3) Many nontaxonomists have too high a regard for the "current taxonomy." Were they more familiar with the meager justification that sometimes supports present taxonomic allocations, they would be far less likely to accept uncritically the judgment of the last taxonomist who happened to revise the group with which they are concerned.

(4) Genetic differentiation in structural genes (the type usually studied by current biochemical techniques) is much more dependent on time since populations have shared a common ancestor than is the rate of change in morphology. Thus the most accurate phylogenetic reconstructions should be based on structural gene evolution. Surprisingly, some cladists (e.g., Farris *et al.*, 1982) seem to be the most negative about the value of biochemical data in phylogenetic reconstruction and appear to be more interested in using Hennigian methods for morphological characters (well known to evolve at different rates in different evolutionary lines). They reject the very characters (products of structural genes) that should provide the best data for estimating the real phylogeny of a group since they apparently evolve at much more constant rates than morphological characters.

(5) Taxonomists have long recognized that the amount of morphological differentiation at the species level is less important in determining taxonomic status than is distinctness (Mayr, 1957). Thus taxonomists are conditioned to look for distinctive features separating sympatric populations as indications of reproductive isolation rather than using the amount of morphological differentiation for that purpose. Systematists not familiar with the new biochemical techniques are understandably cautious concerning genetic distance data that cannot be used as diagnostic or key characters in the same way as standard taxonomic characters. However, electrophoretic data often provide information not only on amount of differentiation but also on whether or not there are fixed genetic differences between sympatric forms (indicating reproductive isolation in nature).

(6) The taxonomic recognition of isolated allopatric populations has always been difficult for the taxonomist because the definitive criterion of reproductive isolation in nature cannot be applied. I have argued (*in* Wake, 1981: 261–262) that in salamanders, which have so few variable

systematic characters and may evolve morphologically at extremely slow rates, genetic distance data may be more useful in determining the taxonomic status of allopatric populations than morphological data. Wake (1981) argues that chaos would result if the amount of genetic divergence, as measured by allozyme data, were used as a basis for making taxonomic decisions on the status of allopatric populations. He also feels the same way about the subdivision of continuously distributed populations into taxonomically recognized units on the basis of genetic distance data (personal communication).

Patterns similar to those seen in the *P. glutinosus* complex have been found in western plethodontid salamanders of the genus *Batrachoseps* (Yanev, 1978, 1980). Yanev discovered several parapatric genetically differentiated forms that have variable amounts of gene exchange in contact zones where their ranges meet or overlap. She recognized these forms as semispecies although she (Yanev, 1980) applies to them the trinomen (nomenclaturally indicating that they are subspecies). However, I agree that the semispecies seems to best indicate the taxonomic relationships of these populations and it is the arrangement I suggest for groups 1–14. Groups 15 and 16 (*P. aureolus* and *P. kentucki*) are widely sympatric with other groups and are not semispecies. Mayr (1963) defines semispecies as "the component species of a superspecies; also populations that have acquired some, but not yet all, attributes of species rank; borderline cases between species and subspecies."

The taxonomic recognition of parapatric or allopatric genetically differentiated forms is often equivocal for several reasons:

(1) There is no established criterion of a specific amount of genetic differentiation that is always associated with the development of reproductive isolation. Some workers, for example Wake (1981), believe that there is as yet no convincing evidence that build-up of genetic distance, as measured by allozyme variation, should alone be used to determine species status, which he believes primarily should be based on morphological data. Although Wake correctly emphasizes the value of electrophoretic data in properly determining the real taxonomic relationships in some instances (e.g., when two morphotypes of a population are genetically identical in allele frequences at all loci and are clearly interbreeding, or when two sibling sympatric forms have fixed genetic differences and are therefore not interbreeding), he (personal communication) does object to making species level determinations of taxonomic status solely on the basis of allozyme data, particularly when the

populations are allopatric. Baverstock *et al.* (1977), Thorpe (1982), and Bullini (1983), however, believe that estimates of genetic distance are probably the best criteria for making taxonomic decisions in doubtful cases. Borderline cases could still be very difficult because the confidence intervals of Nei distances tend to be quite large unless a great many more loci are used than in most current electrophoretic studies. In addition, Uzzell and Wake (personal communication) believe there is a substantial difference in comparisons in which there are merely frequency differences between two populations compared to cases where there are fixed differences, even though the *D* values in the two examples may be the same. The particular mix of fast- and slow-evolving loci (Sarich, 1977) used in a study also has a considerable effect on the magnitude of the *D* values obtained.

(2) The treatment of isolated allopatric populations has always been a difficult problem for the taxonomist. Mayr (1942) indicates that the designation of such populations as species or subspecies is often subjective because the extent of reproductive isolation cannot usually be determined. He suggests that a measure of the amount of morphological divergence that usually is associated with reproductive isolation between good species in each taxonomic group can be estimated by comparing closely related sympatric species of the same group. Brown and Wilson (1956) urge caution in using this measure for allopatric forms because of the likelihood of morphological character displacement in closely related sympatric species. I suggest that genetic distance data may be superior to morphological divergence in aiding the taxonomist to make better species level determinations in such situations.

(3) In many of the 16 *P. glutinosus* groups there are no known diagnostic morphological characters that may be used to identify either living or preserved specimens. However, museum specimens of the other previously recognized species in the group (*yonahlossee*, *jordani*, *ouachitae*, *caddoensis*, and *fourchensis*) are also difficult to distinguish after their diagnostic color pigments have been lost in preservatives. Although the same is true of many of the 16 groups of the *P. glutinosus* complex, that should not preclude their taxonomic recognition. No careful multivariate morphometric study has yet been made on any of these species of large plethodons. The advent of electrophoretic methods of analysis now make it possible not only to identify species but to determine the geographic subgroup within each species with consid-

erable accuracy, but effective use of the method requires living individuals of all of the forms or a complete frozen tissue collection.

(4) The taxonomic recognition of forms that interbreed in narrow contact or overlap zones has long been a difficult one for taxonomists. Although evolutionarily these populations are intermediate between subspecies (freely interbreeding populations of the same species that differ morphologically) and species (reproductively isolated populations), their taxonomic status is often difficult to decide. It is expected that all pairs of gradually diverging populations would be in an intermediate stage of differentiation at some time during their evolution. If a number of populations of the ancestral *P. glutinosus* were all isolated at approximately the same time (as suggested above), then perhaps all might be in this intermediate period at the same time. The category semispecies has been suggested for such parapatric forms that have acquired some but not all the characteristics of species or are borderline cases between species and subspecies (see above). Amadon (1966) distinguishes between allospecies (parapatric species comprising a superspecies) and semispecies (populations that still exchange genes but not as freely as among conspecific populations and thus have only partially completed the process of speciation). Mayr (1963) states that "semispecies are a special kind of species, not a category different from a species." Others, such as Hall and Selander (1973), Dobzhansky *et al.* (1977), and Yanev (1980), call such forms semispecies but refrain from naming them. Hall and Selander (1973) and Barton and Hewitt (1983) argue that strong selection against hybrids or backcross progeny in contact zones is essentially a barrier to gene flow between such parapatric forms, causing these zones to be very narrow. On this basis they regard the forms as species because they are essentially reproductively isolated. At the opposite extreme, Key (1981) argues that reproductive isolation should be defined as the relationship between two forms that do not ever hybridize in nature or whose F_1 hybrids leave no fertile progeny.

Thus in this one complex, previously recognized as a single taxonomic species, we find many of the problems that have troubled taxonomists in defining the limits of species. In previous papers (Highton and MacGregor, 1983; Highton, 1984), four of the groups (1, 6, 15, and 16) have been recognized taxonomically as species because they are sympatric with and reproductively isolated from other groups. The remaining 12 groups replace groups 1 and 6 and each other geograph-

ically. In some narrow contact zones there is evidence of gene exchange, whereas in others, such as those separated by large rivers, fixed differences indicate no gene exchange for a considerable amount of time. No data are yet available for some contact zones, but the amount of genetic divergence is large. In still others there appears to be considerable gene exchange as indicated by several populations with intermediate allele frequencies. Genetic variation across contact zones is now being studied by the writer.

There can be no question about the recognition of *P. kentucki* and *P. aureolus* as distinct species because they are reproductively isolated forms sympatric with other members of the complex. I feel that the remaining groups should be recognized as semispecies. Some taxonomists would probably prefer to recognize them as subspecies and this option is always open to those who believe that the amount of genetic divergence is not sufficient to recognize these forms as species. For those who object to using allozyme data in making taxonomic judgements, groups 1–14 can be referred to collectively as the "*P. glutinosus* complex."

My taxonomic treatment of the *Plethodon glutinosus* complex is the same as that adopted for leopard frogs of the *Rana pipiens* complex. The latter is also comprised of many closely related parapatric and allopatric forms that sometimes hybridize in narrow contact zones. The amount of genetic differentiation, as indicated by call differences and partial reproductive isolation when crossed in the laboratory, is sufficient to support their recognition as species rather than subspecies (Pace, 1974; Frost and Platz, 1983; Hillis *et al.*, 1983; Platz and Frost, 1984; and references therein). Unpublished data on electrophoretic genetic comparisons among the various species of the *R. pipiens* complex by Sage (personal communication) indicate that their genetic distances are in the same range as those of the *P. glutinosus* complex.

Whether or not all of the forms of the *P. glutinosus* complex now have been recognized remains to be determined. As mentioned above, *P. kentucki* has highly differentiated populations that are not analyzed in this paper, and it may be comprised of more than one form. Moreover, in spite of the extensive sampling, some types recognized here could have been missed because of their very small ranges (e.g., groups 7, 10, and 14). Further work could well discover additional forms.

Some of the following synonymies are incomplete in that the first reference to each species as *Plethodon glutinosus* is usually omitted, except in cases where salamanders from the original locality have been studied electrophoretically. If morphometric studies eventually provide characters to distinguish between the species of the *Plethodon glutinosus* complex recognized in this paper, then it should be possible to assign individuals mentioned in the literature to the proper form, provided investigators placed voucher specimens in museum collections.

Group 1.

Plethodon teyahalee Hairston
Southern Appalachian Slimy Salamander

Plethodon glutinosus (Green): Brimley (1912) (part). Highton (1970) (part).
Plethodon jordani teyahalee Hairston (1950:269).
Plethodon jordani Blatchley: Highton (1962b).
Plethodon (glutinosus) glutinosus (Green): Bishop (1941) (part).
P(lethodon). teyahalee Hairston: Highton (1984).

Holotype: UMMZ 100807, an adult male collected 23 August 1949 by Nelson G. Hairston, on Teyahalee Bald (=Johanna Bald), at an elevation of 1,380 m in the Snowbird Mountains, Graham-Cherokee county line, North Carolina.

Diagnosis: A large, light-chinned species with very small white dorsal spots, reduced lateral white spotting, and often with small red spots on the legs. The unique combination of genetic alleles that distinguishes *P. teyahalee* from other species of the *P. glutinosus* group is shown in Table 4. Pgi allele *c* and Trf allele *a* are characteristic of *P. teyahalee* populations but are usually rare or absent in the other species.

Distribution: West of the French Broad River in the Blue Ridge physiographic province of southwestern North Carolina and in immediately adjacent Tennessee. It also occurs in northern Rabun County, Georgia, and in Oconee, Pickens, Anderson, and Abbeville counties, South Carolina (Fig. 8).

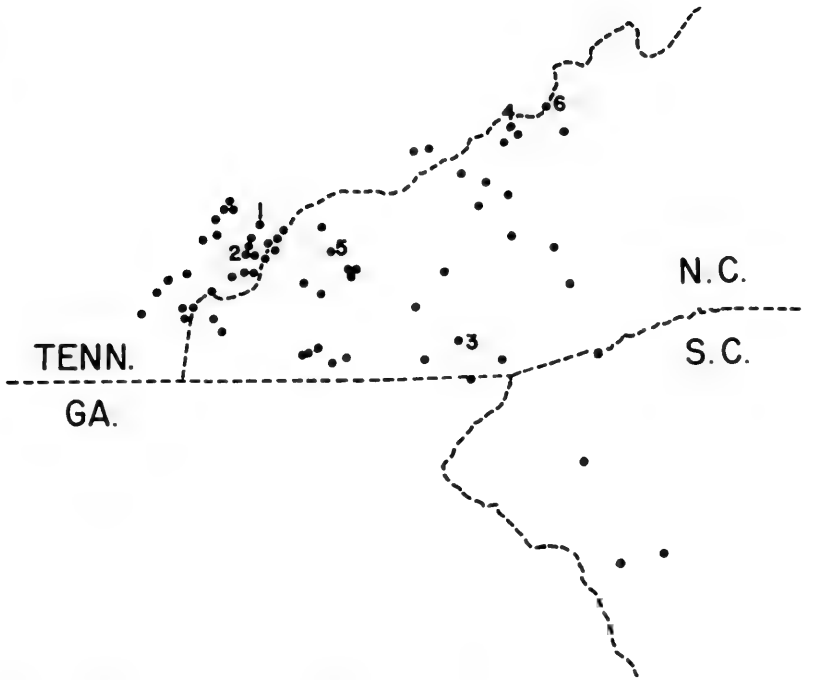

Fig. 8. Distribution of *P. teyahalee* (Group 1) in the southern Appalachian Mountains based on samples identified to species electrophoretically.

Group 2.

Plethodon chattahoochee Highton, new species
Chattahoochee Slimy Salamander

Plethodon shermani Brimley: Howell (1909).
Plethodon glutinosus (Green): Bailey (1937) (part). Highton (1970) (part).
Plethodon shermani rabunensis Pope and Hairston: Hairston and Pope (1948) (part).
Plethodon jordani rabunensis Pope and Hairston: Hairston (1950) (part).
Plethodon jordani Blatchley: Highton (1962b) (part).
Plethodon glutinosus glutinosus (Green): Highton (1962b) (part).

Holotype: USNM 168527, an adult male collected 22 July 1961 by Richard Highton and Thomas Savage, at locality 9 (Fig. 1, Appendix 1),

0.3 km east of the top of Brasstown Bald, at an elevation of 1,353 m, Towns County, Georgia.

Allotype: USNM 168518, an adult female, same data as holotype.

Paratypes: USNM 168519-26, 168528-35; same data as holotype.

Other material: Specimens from localities indicated in Figure 9 have been identified genetically as *P. chattahoochee*.

Diagnosis: A large, light-chinned species with little or no dorsal spotting (except for populations in the extreme western part of its range) and abundant white or yellow lateral spotting. The unique combination of genetic alleles that distinguishes *P. chattahoochee* from other species of the *P. glutinosus* group is shown in Table 4. Allele *c* at the Pt-2 locus is unique to this species.

Description of Holotype: After preservation: lengths from tip of snout to anterior angle of vent, 59 mm (in life, 62 mm); to posterior angle of vent, 64 mm; total length, 115 mm. Head length (snout to gular fold) 14 mm; head width at widest point, 9 mm; length of front limbs, 14 mm, and of hind limbs, 15 mm. Vomerine teeth number 7 on both sides. 16 costal grooves (equivalent to 17 trunk vertebrae). Coloration in life: no white or brassy spotting on the black dorsum; numerous yellow spots on sides and a few small yellow spots on chin and underside of legs; few tiny red spots on dorsal surface of front limbs; venter lighter than ground color of back and sides; three small yellow spots on anterior venter; a few tiny brassy flecks on both eyelids and one small white spot on right eyelid; a trace of brassy flecking in iris of both eyes. Chin lighter than venter and with a prominent, round mental gland. Many tiny spots (hedonic glands) on ventral surface of body and tail.

Description of Allotype: After preservation: length from tip of snout to anterior angle of vent, 60 mm (in life, 64 mm), to posterior angle of vent, 65 mm; total length, 132 mm; head length, 14 mm; head width, 9 mm; length of front limbs, 13 mm, and of hind limbs, 16 mm. Vomerine teeth number 8 on the right and 9 on the left. 16 costal grooves. Coloration in life: similar to holotype except no red spots on legs, no yellow spots on venter, and no brassy flecks in iris.

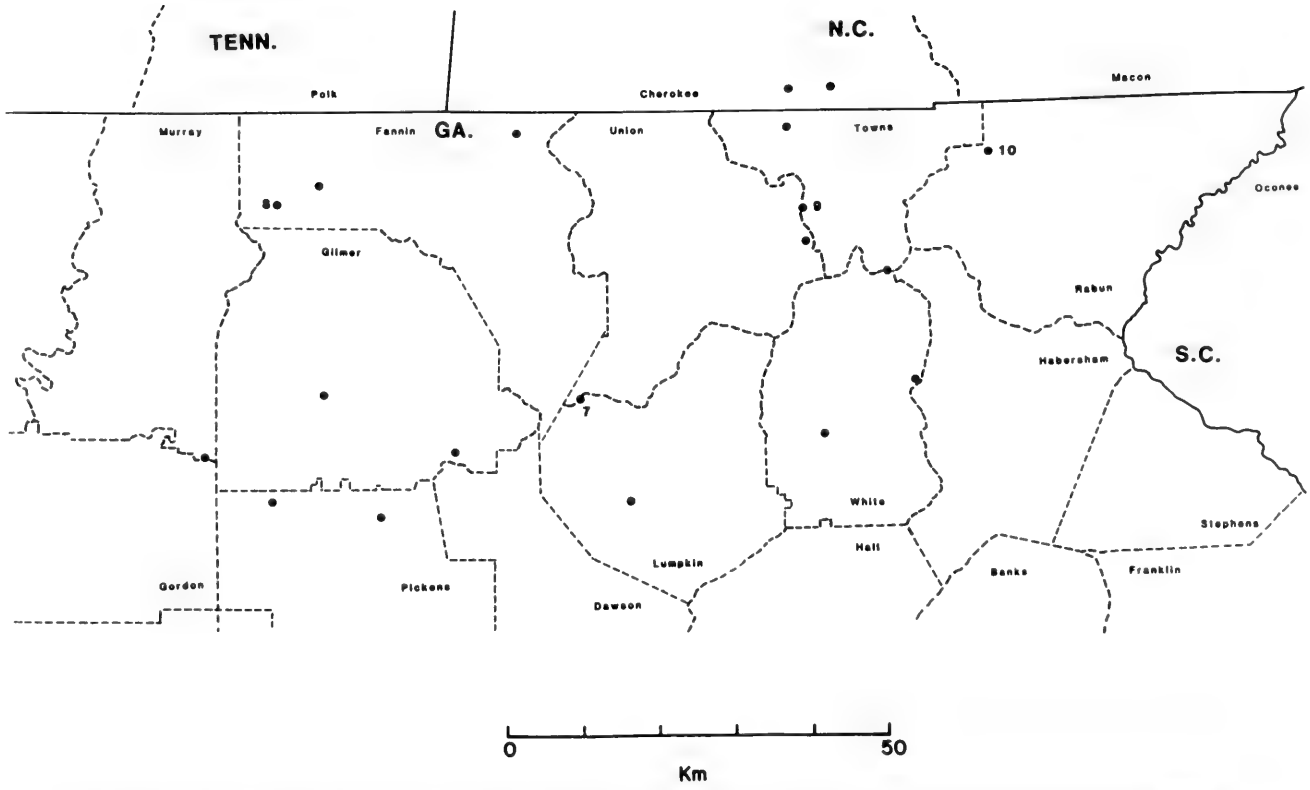

Fig. 9. Distribution of *P. chattahoochee* (Group 2) in northern Georgia and southwestern North Carolina based on samples identified to species electrophoretically. Counties are indicated.

Variation in the Type Series: In life, 9 of 18 specimens in the type series had small red spots on anterior legs; only the smallest (22 mm snout-anterior angle of vent) had a red spot on the dorsum. None had any red pigment on the hind limbs, but one had one tiny red spot on one cheek. The juvenile had several brassy flecks on the anterior dorsum, but only two of the larger animals had traces of dorsal brassy flecking (one only on the head, the other only on the dorsum). All had yellow spotting on the sides and undersides of the limbs. Twelve of the 18 had yellow spotting on the chin and 8 also had similar spotting on the anterior venter. All but the allotype had brassy flecking in the irises and all but one had brassy flecking on the eyelids.

Distribution: Most of the Blue Ridge physiographic province of northern Georgia (Fig. 9). It also occurs in southeastern Cherokee County, North Carolina. The distribution of this species approximately coincides with the limits of the Chattahoochee National Forest, after which the species is named.

Group 3.

Plethodon chlorobryonis Mittleman, new status
Atlantic Coastal Slimy Salamander

Plethodon glutinosus chlorobryonis Mittleman (1951:108).

Holotype: USNM 129933, an adult male collected 10 November 1950 by Myron B. Mittleman and C. B. Goodstein, at or near locality 14 (Fig. 1, Appendix 1), 13 miles north of New Bern, Craven County, North Carolina.

Diagnosis: A small-sized species (except that non-coastal plain populations tend to be larger) with very small, slightly brassy, white dorsal spots and abundant lateral white or yellow spotting. The unique combination of genetic alleles that distinguishes *P. chlorobryonis* from other species of the *P. glutinosus* group is shown in Table 4.

Distribution: Coastal Plain physiographic province of southeastern Virginia, North Carolina, and northeastern South Carolina. It enters the Piedmont physiographic province in southeastern Virginia, central and western South Carolina, and the Blue Ridge physiographic province in northeastern Georgia (Fig. 10).

Fig. 10. Distribution of of *P. chlorobryonis* (Group 3) in the middle Atlantic states based on samples identified to species electrophoretically.

Group 4.

Plethodon variolatus (Gilliams), new combination
South Carolina Coastal Slimy Salamander

Salamandra variolata Gilliams (1818:460).
Plethodon variolosum (Gilliams). Duméril, Bibron, and Duméril (1854).

Holotype: Not known to exist. The type locality is the "southern states."

Schmidt (1953) restricted the type locality to the vicinity of Charleston, South Carolina, but Neill (1957) criticized Schmidt's restriction of this and other type localities. To remove future question as to which species this name should apply, I designate UNSM 267104 as the neotype of *Salamandra variolata*, an adult male collected 27 March 1986 by David E. Carr, from locality 27 (Fig. 1, Appendix 1), at an elevation of 6 m, Beechtree Recreation Area, Berkeley County, South Carolina.

Description of Neotype: In life: length from tip of snout to anterior angle of vent, 57 mm, and to posterior angle of vent, 61 mm; total length, 121 mm; head length, 13 mm; head width, 10 mm; length of front limbs, 13 mm, and of hind limbs, 14 mm. (After preservation, snout-vent lengths 52 and 56 mm and total length 114 mm.) Vomerine teeth number 8 on the right and 7 on the left. 16 costal grooves. A few small white dorsal spots, each with scattered brassy flecking, especially at edges of spots. Abundant large yellow spots on sides; few scattered yellow spots on chin and venter. Venter lighter than black ground color of dorsum; chin much lighter than venter and with a small round mental gland.

Diagnosis: A small species with small to medium-sized dorsal brassy white spots and abundant lateral white or yellow spotting. Populations from Jasper County, South Carolina, lack the lateral and dorsal spotting, as do occasional specimens from other populations. The unique combination of genetic alleles that distinguishes *P. variolatus* from other species of the *P. glutinosus* group is shown in Table 4.

Distribution: Southern Coastal Plain physiographic province of South Carolina and extreme southeastern Georgia.

Group 5.

Plethodon ocmulgee Highton, new species
Central Georgia Slimy Salamander

Holotype: USNM 257426, an adult male collected 8 November 1976 by S. Blair Hedges and Richard Highton, at locality 32 (Fig. 1, Appendix 1), Little Ocmulgee State Park, at an elevation of 49 m, Wheeler County, Georgia.

Allotype: USNM 257427, an adult female, same data as holotype.

Paratypes: USNM 257428-257464, same data as holotype.

Diagnosis: A small-sized species (except for Piedmont populations) with few small brassy dorsal spots and a moderate amount of lateral white spotting. The unique combination of genetic alleles that distinguishes *P. ocmulgee* from other species of the *P. glutinosus* group is shown in Table 4. Allele *a* of the Gdh locus, rare or absent from all other species, is always common in *P. ocmulgee*.

Description of Holotype: After preservation: length from tip of snout to anterior angle of vent, 52 mm (in life, 56 mm) and to posterior angle of vent, 57 mm; total length, 103 mm. Head length (snout to gular fold), 13 mm; head width at widest point, 8 mm; length of front limbs, 12 mm, and of hind limbs, 13 mm. Vomerine teeth number 4 on the right and 7 on the left. 16 costal grooves. Few small brassy and white dorsal spots in life and numerous white spots on sides; venter almost as dark as black ground color of back and sides, chin slightly lighter than venter; many tiny spots (hedonic glands) on ventral surface of the body and tail; a small round mental gland.

Description of Allotype: After preservation: length from tip of snout to anterior angle of vent, 59 mm (in life, 62 mm) and to posterior angle of vent, 64 mm; total length, 119 mm; head length, 15 mm; head width, 9 mm; length of front limbs, 13 mm on the right and 14 mm on the left; and of hind limbs, 15 mm. Vomerine teeth number 8 on each side. 16 costal grooves. Coloration in life similar to holotype.

Distribution: The upper Coastal Plain and adjacent Piedmont physiographic provinces of central Georgia. Much of its range is in the Ocmulgee River drainage, after which the species is named.

Group 6.

Plethodon glutinosus (Green)
Northern Slimy Salamander

Salamandra glutinosa Green (1818:357).
Plethodon glutinosus (Green): Tschudi (1838).
Plethodon glutinosum (Green): Gray (1850).

Cylindrosoma glutinosum (Green): Duméril, Bibron, and Duméril (1854).
Salamandra melanoleuca Wied (1865:130–131). Type locality: Nazareth, Pennsylvania.
Plethodon glutinosus glutinosus (Green): Dunn (1920). Bishop (1943). Grobman (1944).

Holotype: Dunn (1926) states that the holotype is not known to exist, but the type locality is probably Princeton, New Jersey.

Diagnosis: A large species with large brassy colored dorsal spots and moderately abundant lateral white or yellow spotting. It differs from sympatric *P. kentucki* in having a darker chin, smaller mental gland in adult males, and larger, more brassy dorsal spots. It is not detectably different morphologically from groups 7, 10, and 13. The unique combination of genetic alleles that distinguishes *P. glutinosus* from other species of the *P. glutinosus* group is shown in Table 4.

Distribution: Southwestern Connecticut west to southern Illinois and south through West Virginia, western Virginia, Kentucky and Tennessee to eastern Alabama and northwestern Georgia (Fig. 11).

Group 7.

Plethodon kiamichi Highton, new species
Kiamichi Slimy Salamander

Holotype: USNM 257314, an adult male collected 5 June 1973 by Wayne Garber, Richard Highton, and James Hook, at locality 76 (Fig. 1, Appendix 1), Round Mountain, at an elevation of 640 m, LeFlore County, Oklahoma.

Allotype: USNM 257315, an adult female, same data as the holotype.

Paratypes: USNM 257316-257347, same data as the holotype.

Other material: Specimens from localities indicated in Figure 12 have been identified genetically as *P. kiamichi*.

Diagnosis: A large species with large brassy dorsal spots and moderately abundant lateral white or yellow spotting. It is not detectably different

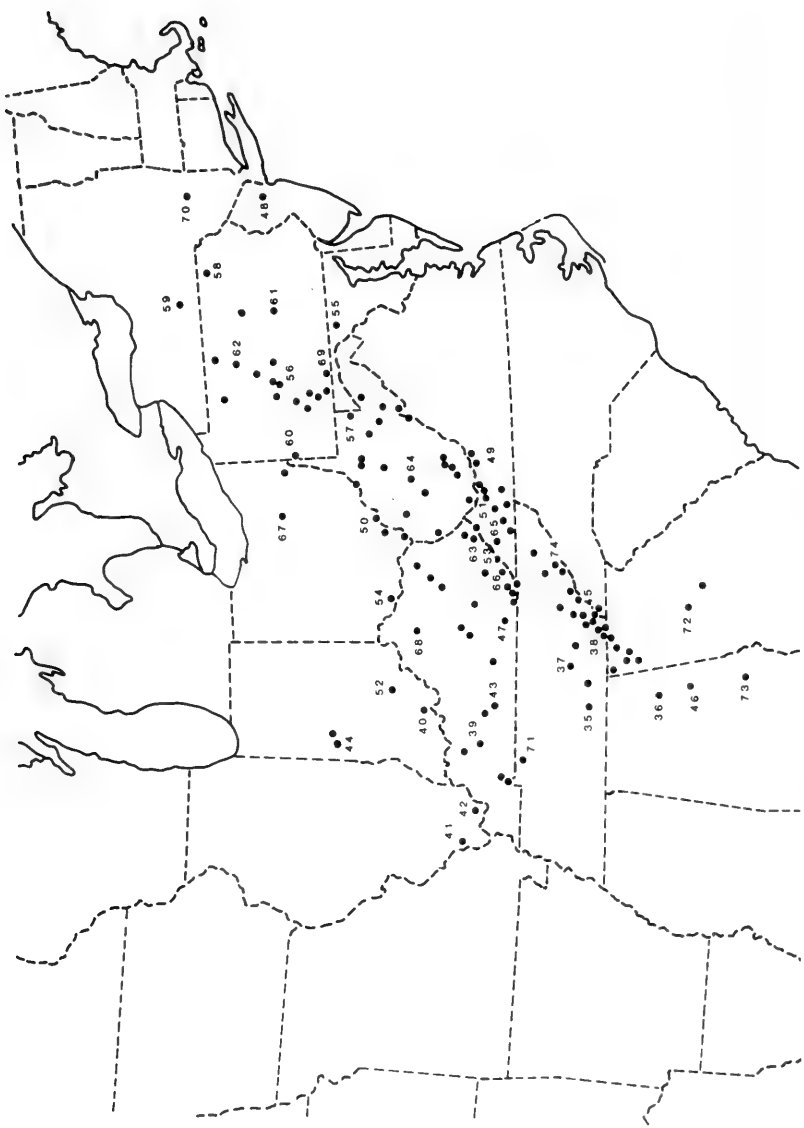

Fig. 11. Distribution of *P. glutinosus* (Group 6) in the eastern United States based on samples identified to species electrophoretically.

morphologically from groups 6, 10, and 13. The unique combination of genetic alleles that distinguishes *P. kiamichi* from other species of the *P. glutinosus* group is shown in Table 4. Got-1 allele *b*, common in *P. kiamichi*, is absent from the other species of the *P. glutinosus* complex, but is also common in *P. ouachitae* (Table 4).

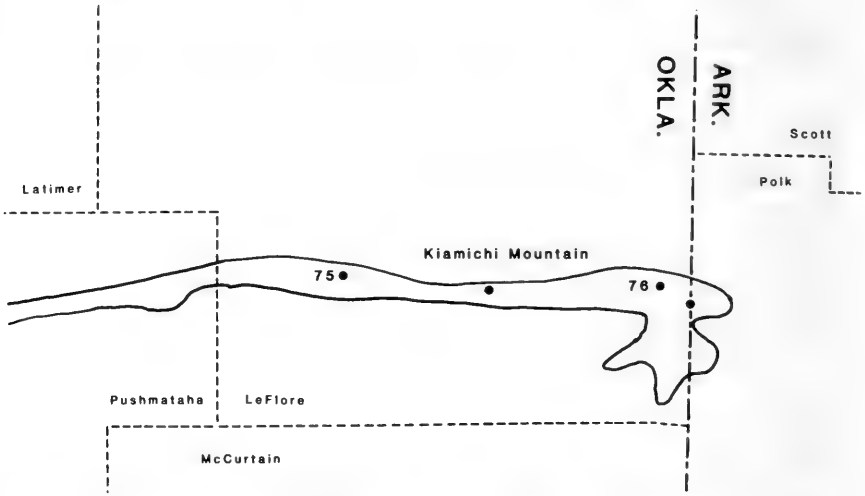

Fig. 12. Distribution of *P. kiamichi* (Group 7) on Kiamichi Mountain, Arkansas and Oklahoma, based on samples identified to species electrophoretically. Counties are indicated.

Description of Holotype: After preservation: length from tip of snout to anterior angle of vent, 65 mm (in life, 69 mm), and to posterior angle of vent, 70 mm; total length, 141 mm; head length (snout to gular fold), 15 mm; head width at widest point, 10 mm; length of front limbs, 15 mm, and of hind limbs, 16 (right) and 15 mm (left). Vomerine teeth number 13 on the right and 11 on the left. 16 costal grooves. Many small white dorsal spots with associated brassy flecking in life; numerous white spots on sides, chin, and legs; venter dark as ground color of back and sides and with few white spots; chin as dark as venter with a prominent, large, oval-shaped mental gland. Many tiny spots (hedonic glands) on ventral surface of body and tail. Few tiny brassy flecks and white spots on the eyelids in life.

Description of Allotype: After preservation: length from tip of snout to anterior angle of vent, 79 mm (in life, 83 mm), and to posterior angle of vent, 85 mm; total length, 160 mm; head length, 17 mm; head width, 11

mm; length of front limbs, 15 mm, and of hind limbs, 17 mm. Vomerine teeth number 9 on the right and 8 on the left. 17 costal grooves. Coloration in life similar to holotype except for absence of ventral hedonic glands.

Distribution: Known only from Round and Kiamichi mountains in Polk County, Arkansas, and LeFlore County, Oklahoma (Fig. 12). This species is named for Kiamichi Mountain.

Group 8.

Plethodon mississippi Highton, new species
Mississippi Slimy Salamander

Holotype: USNM 257388, an adult male collected 18 January 1986 by David E. Carr and Richard Highton, at locality 79 (Fig. 1, Appendix 1), Tishomingo State Park, at an elevation of 177 m, Tishomingo County, Mississippi.

Allotype: USNM 257389, an adult female, same data as the holotype.

Paratypes: USNM 257390-257425, same locality as the holotype.

Other material: Specimens from localities indicated in Figure 13 have been identified genetically as *P. mississippi*.

Diagnosis: A small species (except for populations in the Appalachian Plateau of northern Alabama that reach a larger size) with large brassy dorsal spots and abundant lateral white or yellow spotting. Morphologically, it is not detectably different from Group 11. The unique combination of genetic alleles that distinguishes *P. mississippi* from other species of the *P. glutinosus* group is shown in Table 4.

Description of Holotype: In life: length from tip of snout to anterior angle of vent, 65 mm, and to posterior angle of vent, 69 mm; total length, 137 mm; head length, 15 mm; head width, 10 mm; length of front limbs, 15 mm, and of hind limbs, 16 mm. Vomerine teeth number 10 on the right and 11 on the left. 16 costal grooves. Many small white spots with slight brassy flecking on black dorsum; numerous yellow spots on sides; few small white spots on chin and legs; belly as dark as ground

color of back and sides and chin slightly lighter than belly with a round mental gland. Many tiny spots (hedonic glands) on ventral surface of body and tail. Few tiny brassy flecks and small white spots on eyelids.

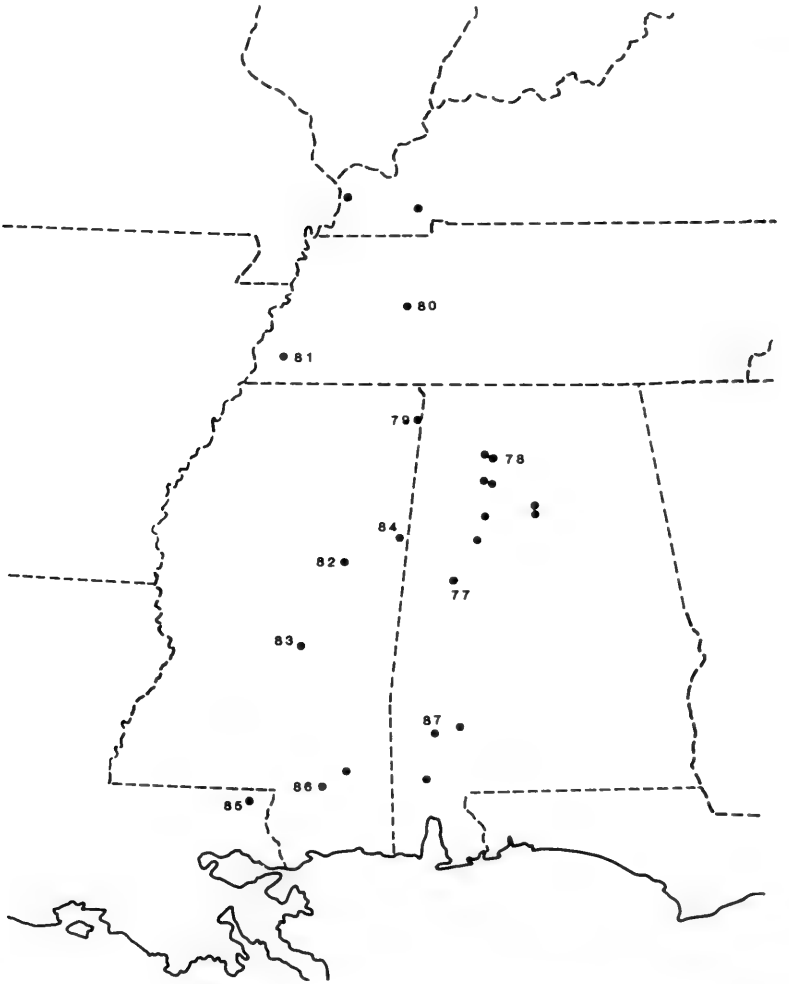

Fig. 13. Distribution of *P. mississippi* (Group 8) in the south-central United States based on samples identified to species electrophoretically.

Description of Allotype: After preservation: length from tip of snout to anterior angle of vent, 66 mm (in life, 71 mm), and to posterior angle of

vent, 71 mm; total length, 142 mm; head length, 15 mm; head width, 10 mm; length of front limbs, 14 mm; and of hind limbs, 16 mm. Vomerine teeth number 7 on the right and 9 on the left. 16 costal grooves.

Distribution: Western Alabama, Mississippi, Florida Parishes of southeastern Louisiana, western Tennessee, and western Kentucky (Fig. 13). The species is named for the State of Mississippi.

Group 9.

Plethodon kisatchie Highton, new species
Louisiana Slimy Salamander

Holotype: USNM 257348, an adult male collected 27 January 1971 by Richard Highton, James Hook, George Kramer, Mark Mello, Donald C. Morizot, and David Walter, at locality 90 (Fig. 1, Appendix 1), along Indian Creek, at an elevation of 30 m, Grant Parish, Louisiana.

Allotype: USNM 257349, an adult female, from locality 89 (Fig. 1, Appendix 1), Winn Parish, Louisiana, collected on 16 December 1973 by Richard Highton and Scott Highton.

Paratypes: USNM 257350-257355, same locality as the holotype; USNM 257356- 257387, same locality as the allotype.

Diagnosis: A small species with very large brassy dorsal spots. The unique combination of genetic alleles that distinguishes *P. kisatchie* from other species of the *P. glutinosus* group is shown in Table 4. Est allele *l* is usually rare or absent in the other species.

Description of Holotype: After preservation: length from tip of snout to anterior angle of vent, 52 mm (in life, 56 mm), and to posterior angle of vent, 56 mm; total length, 115 mm; head length, 13 mm; head width, 8 mm; length of front limbs, 12 mm, and of hind limbs, 13 mm. Vomerine teeth number 7 on the right and 8 on the left. 16 costal grooves. Coloration in life: many large brassy white spots on black dorsum; numerous yellow spots on sides and legs but none on chin and venter; venter slightly lighter than ground color of back and sides; chin slightly lighter than venter; chin with small round mental gland; many tiny spots (hedonic glands) on ventral surface of body and tail; a few tiny

brassy flecks and small white spots on eyelids. Areas free of black pigmentation along anterior margin of gular fold, ventral margin of limb insertions, and front limb joints.

Description of Allotype: After preservation: length from tip of snout to anterior angle of vent, 58 mm (in life, 60 mm), and to posterior angle of vent, 63 mm; total length, 125 mm; head length, 14 mm; head width, 9 mm; length of front limbs, 12 mm, and of hind limbs, 14 mm. Vomerine teeth number 6 on the right and 5 on the left. 16 costal grooves. Coloration in life: similar to holotype except no hedonic glands on venter and reduced pigmentation (not absence) at hind limb insertions; a few small yellow spots on the chin and belly.

Distribution: From central Louisiana north to southern Arkansas. The species is named for the Kisatchie National Forest.

Group 10.

Plethodon sequoyah Highton, new species
Southeastern Oklahoma Slimy Salamander

Holotype: USNM 257485, an adult male collected 11 October 1976 by S. Blair Hedges and Richard Highton, at locality 91 (Fig. 1, Appendix 1), Beavers Bend State Park, at an elevation of 140 m, McCurtain County, Oklahoma.

Allotype: USNM 257486, an adult female, same locality as the holotype, collected on 25 May 1978 by Richard Highton, Scott Highton and Jeffrey Streicher.

Paratypes: USNM 257487-257521, same data as the allotype.

Diagnosis: A large species with large brassy dorsal spots and moderately abundant white or yellow spotting. It is not detectably different morphologically from groups 6, 7, and 13. The unique combination of genetic alleles that distinguishes *P. sequoyah* from other species of the *P. glutinosus* group is shown in Table 4. Mdh-2 allele *a* is unique to *P. sequoyah*.

Description of Holotype: After preservation: length from tip of snout to

anterior angle of vent, 58 mm (in life, 61 mm), and to posterior angle of vent, 63 mm; total length 133 mm; head length, 14 mm; head width, 9 mm; length of front limbs, 14 mm; and of hind limbs, 15 mm. Vomerine teeth number 8 on the right and 9 on the left. 15 costal grooves. Numerous small white brassy dorsal spots; larger yellow lateral spots; chin with white spots; venter almost as dark as black ground color of back and sides, chin slightly lighter than venter with small, rounded, mental gland.

Description of Allotype: After preservation: length from tip of snout to anterior angle of vent, 64 mm (in life, 69 mm), and to posterior angle of vent, 69 mm; total length, 136 mm; head length, 15 mm; head width, 10 mm; length of front limbs, 14 mm, and of hind limbs, 15 mm. Vomerine teeth number 10 on the right and 9 on the left. 16 costal grooves. Coloration in life similar to holotype.

Distribution: Known only from the type locality, Beavers Bend State Park, McCurtain County, Oklahoma. Named for the Oklahoma Indian Sequoyah, who devised the Cherokee alphabet.

Group 11

Plethodon grobmani Allen and Neill, new status
Southeastern Slimy Salamander

Plethodon glutinosus grobmani Allen and Neill (1949:112).

Holotype: ERA-WTN 19220, an adult female collected 1 October 1949 by E. Ross Allen, Bobby Allen, and Wilfred T. Neill, at Half-mile Creek Swamp, about ½ mile northeast of Silver Springs, Marion County, Florida.

Diagnosis: A small species with large brassy dorsal spots and abundant lateral white or yellow spotting. Morphologically, it is not detectably different from Group 8. The unique combination of genetic alleles that distinguishes *P. grobmani* from other species of the *P. glutinosus* group is shown in Table 4.

Distribution: Southern Alabama and southern Georgia south to central Florida (Fig. 14). Topotypes are genetically similar to other populations from the Florida peninsula.

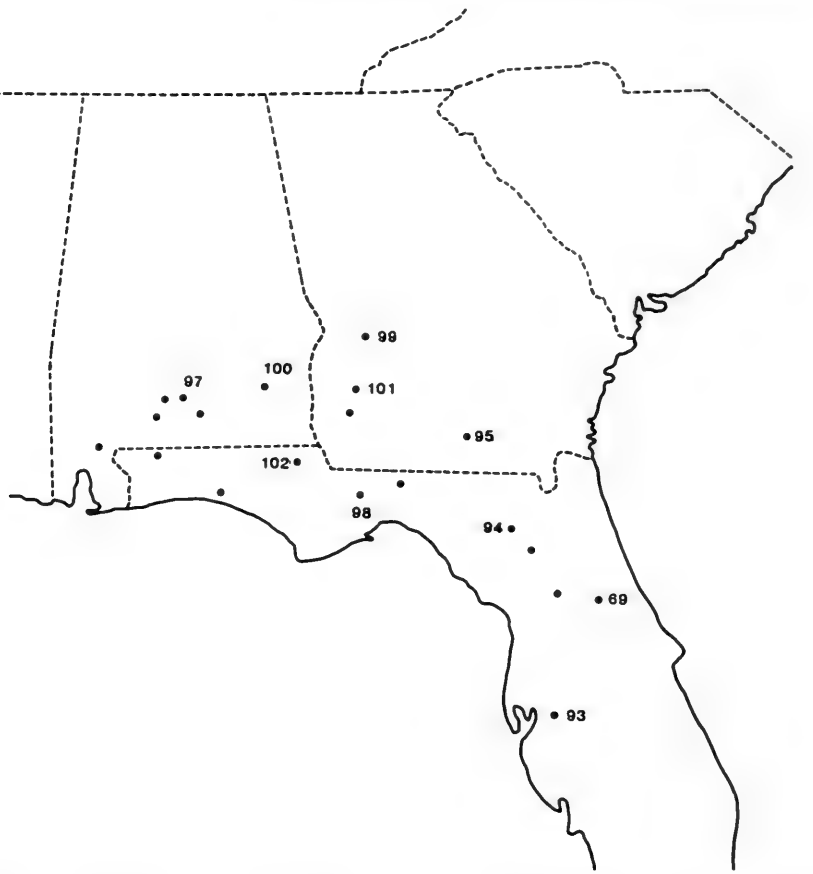

Fig. 14. Distribution of *P. grobmani* (Group 11) in the southeastern United States based on samples identified to species electrophoretically.

Group 12

Plethodon cylindraceus (Harlan), new combination
White-Spotted Slimy Salamander

Salamandra cylindracea Harlan (1825:156–157).

Holotype: Not known to exist. The type locality is South Carolina, probably in the vicinity of Camden, although Schmidt (1953) restricted

the type locality to the vicinity of Charleston, South Carolina. Highton (1962b) pointed out that Camden is more likely the type locality than Charleston. Because Camden is in or near the contact zone between groups 3 and 12, I believe a neotype should be designated to avoid future confusion as to which name applies to this species. I therefore designate USNM 257522 as the neotype of *Salamandra cylindracea*, an adult female collected 31 March 1971 by Richard Highton and Donald C. Morizot, from locality 112 (Fig. 1, Appendix 1), at an elevation of 137 m, Chester County, South Carolina.

Description of Neotype: After preservation: length from tip of snout to anterior angle of vent, 70 mm (in life, 76 mm), and to posterior angle of vent, 74 mm; total length 147 mm; head length, 16 mm; head width, 11 mm; length of front limbs, 14 mm; and of hind limbs, 18 mm. Vomerine teeth number 9 on the right and 7 on the left. 16 costal grooves. Dorsum and legs with small white spots in life and sides with larger yellow spots; a few small yellow spots on cheeks, chin, and venter. Venter and chin slightly lighter than black ground color of dorsum.

Diagnosis: A large, light-chinned species with large white dorsal spots and moderately abundant lateral white spots. The unique combination of genetic alleles that distinguishes *P. cylindraceus* from other species of the *P. glutinosus* group is shown in Table 4. Mdh-1 allele *a*, common in the northern populations of *P. cylindraceus*, is rare or absent in the other species.

Distribution: The Piedmont and Blue Ridge physiographic provinces of Virginia and North Carolina west to the French Broad River and south to the northern Piedmont of South Carolina. It also occurs in parts of the Valley and Ridge physiographic province in western Virginia and extreme eastern West Virginia and in a small area of the Coastal Plain physiographic province of eastern Virginia (Fig. 15).

Group 13.

Plethodon albagula Grobman, new status
Western Slimy Salamander

Plethodon glutinosus albagula Grobman (1944:283).

Holotype: CM 9652, an adult male collected 24 February 1935 by Wesley

Clanton, 20 miles north of San Antonio, Bexar County, Texas.

Diagnosis: A large species with large brassy dorsal spots and moderately abundant lateral white or yellow spotting. Morphologically, it is not detectably different from groups 6, 7, and 10. The unique combination of genetic alleles that distinguishes *P. albagula* from other species of the *P. glutinosus* group is shown in Table 4. The null allele (*h*) in the Pt-2 locus is found only in *P. albagula*.

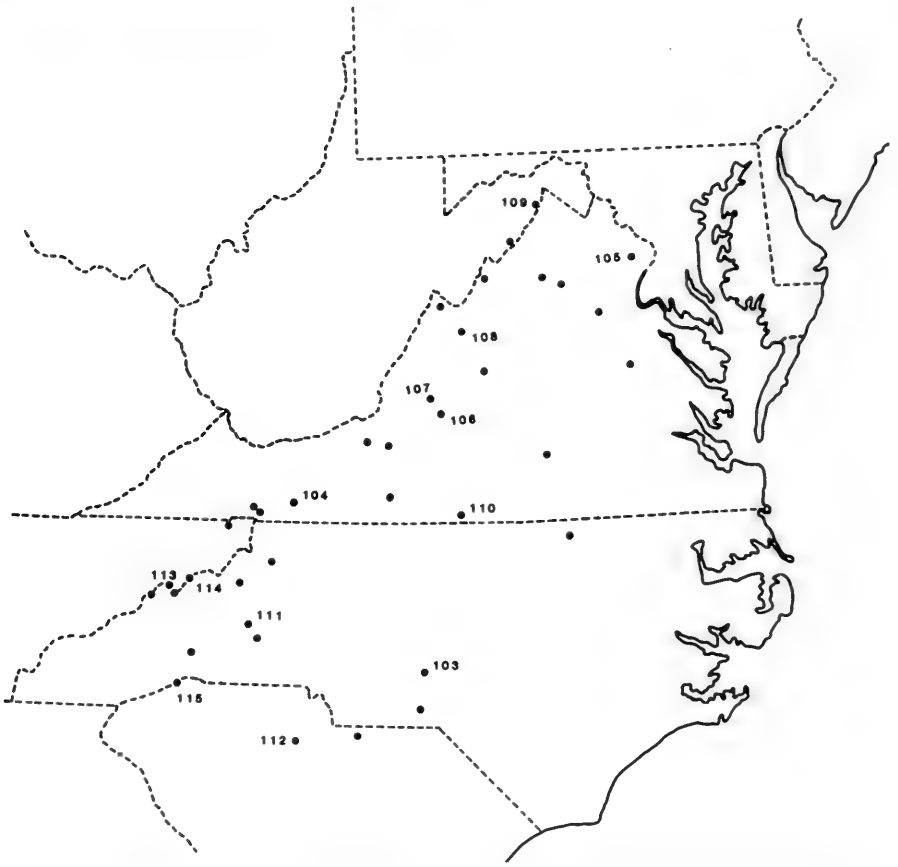

Fig. 15. Distribution of *P. cylindraceus* (Group 12) in the middle Atlantic states based on samples identified to species electrophoretically.

Distribution: Southern Missouri, the highlands of northern and western

Arkansas and eastern Oklahoma exclusive of the ranges of groups 7 and 10; the Balcones Escarpment area of south-central Texas. Additional samples assigned genetically to this species are from Independence, Johnson, Logan, Polk, Scott, and Van Buren counties, Arkansas, and Warren County, Missouri (north of the Missouri River).

Group 14.

Plethodon savannah Highton, new species
Eastern Georgia Slimy Salamander

Holotype: USNM 257465, an adult male collected 17 March 1985 by Richard Highton and Hansjürg Hotz, at locality 128 (Fig. 1, Appendix 1), at an elevation of 101 m, Richmond County, Georgia.

Allotype: USNM 257466, an adult female, same data as the holotype.

Paratypes: USNM 257467-257480, same data as the holotype; USNM 257481-257484 topotypes.

Other material: Specimens from localities indicated in Figure 16 have been identified genetically as *P. savannah*.

Diagnosis: A small species with very little brassy pigment in the white dorsal spots and with abundant lateral white or yellow spots. The unique combination of genetic alleles that distinguishes *P. savannah* from the other species of the *P. glutinosus* group is shown in Table 4. Idh-1 allele *d* is usually rare or absent in the other species.

Description of Holotype: In life: length from tip of snout to anterior angle of vent, 67 mm, and to posterior angle of vent, 72 mm; total length, 139 mm; head length, 15 mm; head width, 11 mm; length of front limbs, 13 mm; and of hind limbs, 16 mm. Vomerine teeth number 9 on the right and 10 on the left. 16 costal grooves. Coloration in life: many white iridophore dorsal spots scattered on the black ground color; abundant white iridophore spotting on sides, dorsal surfaces of limbs, and moderate amount on cheeks, chin and sides of head; venter with few white spots.

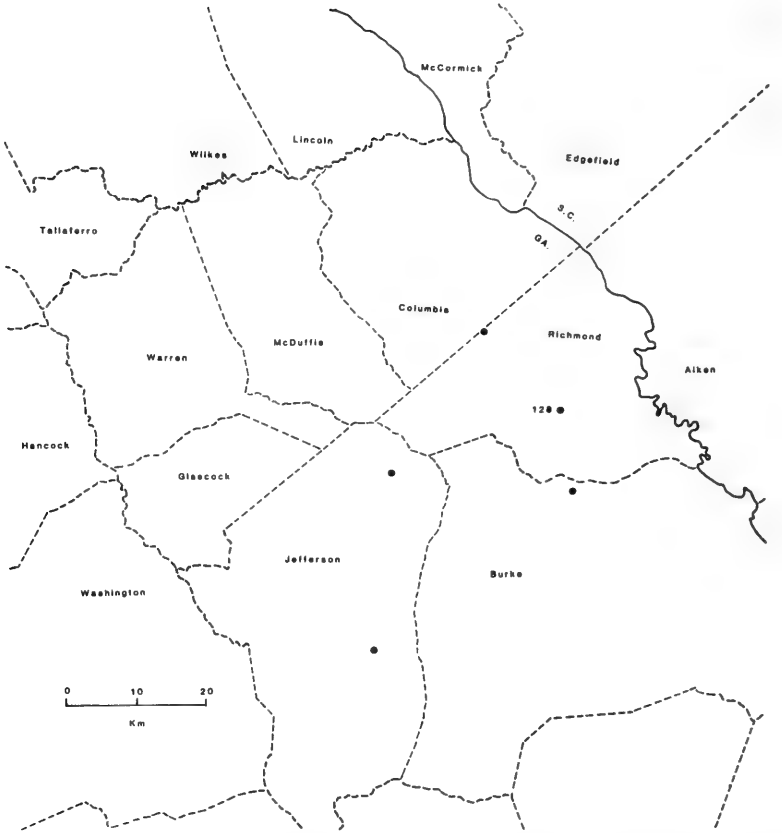

Fig. 16. Distribution of *P. savannah* (Group 14) in eastern Georgia based on samples identified to species electrophoretically. Counties are indicated.

Description of Allotype: In life: length from tip of snout to anterior angle of vent, 65 mm, and to posterior angle of vent, 70 mm; total length, 130 mm; head length, 14 mm; head width, 10 mm; length of front limbs, 13 mm; and of hind limbs, 16 mm. Vomerine teeth number 6 on the right and 7 on the left. 16 costal grooves. Coloration similar to holotype.

Distribution: Known only from Burke, Jefferson, and Richmond counties, Georgia (Fig. 16). The apparent eastern limit of the range is the Savannah River, after which the species is named.

Group 15.

Plethodon aureolus Highton
Tellico Salamander

Plethodon aureolus Highton (1984:2).

Holotype: USNM 238341, an adult male collected 30 June 1979 by Richard Highton and Jeffrey K. Streicher, at Farr Gap (locality 130; Fig. 1; Appendix 1), at an elevation of 872 m, Monroe County, Tennessee.

Diagnosis: The smallest species of the *P. glutinosus* complex with a light chin, abundant dorsal brassy spots, and abundant lateral white or yellow spotting. The unique combination of genetic alleles that distinguishes *P. aureolus* from other species of the *P. glutinosus* group is shown in Table 4. Allele *d* at the Pt-2 locus is unique to *P. aureolus*. Allele *f* at the Ldh-m locus and Idh-2 allele *d* are usually rare or absent in the other species.

Distribution: Between the Little Tennessee and Hiwassee rivers on the western slopes of the Unicoi Mountains in northeastern Polk and Monroe counties, Tennessee, and in western Graham and northwestern Cherokee counties, North Carolina (Highton, 1984: Fig. 1).

Group 16.

Plethodon kentucki Mittleman
Cumberland Plateau Woodland Salamander

Plethodon kentucki Mittleman (1951:105). Highton and MacGregor (1983).
Plethodon jordani kentucki: Schmidt (1953).

Holotype: USNM 129937, an adult male collected in August 1933 by W. Cornett, on Pine Mountain, at an elevation of about 610 m, Harlan County, Kentucky.

Diagnosis: Geographically variable in size but generally smaller than sympatric *P. glutinosus* (Group 6). It usually has a lighter chin than sympatric *P. glutinosus* and its dorsal spots are smaller and possess less brassy pigment. The mental gland of adult males is larger than that of sympatric *P. glutinosus*. The unique combination of genetic alleles that

distinguishes *P. kentucki* from other species of the *P. glutinosus* group is shown in Table 4. Allele *g* of the Pt-2 locus is found only in *P. kentucki*, and Alb alleles *a* and *b*, Trf allele *m*, Got-1 alleles *a* and *d*, and Got-2 allele *d* are usually rare or absent in the other species of the *P. glutinosus* complex.

Distribution: The Cumberland Plateau of western West Virginia west of the New River, eastern Kentucky, southwestern Virginia, and northeastern Tennessee (Fig. 17).

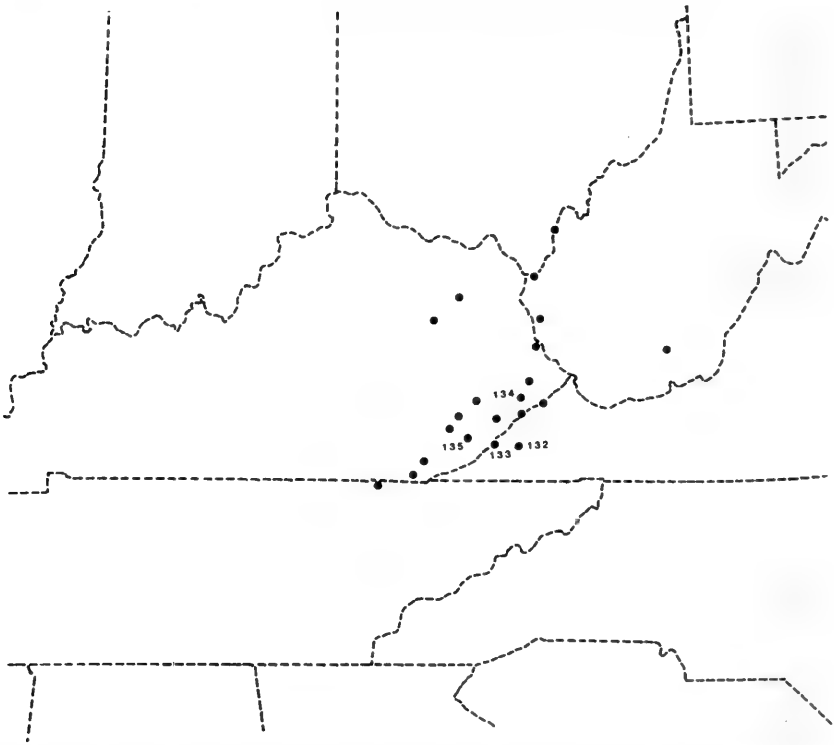

Fig. 17. Distribution of *P. kentucki* (Group 16) in the Cumberland Plateau based on samples identified to species electrophoretically.

SUMMARY

Genetic variation in 22 independent loci of *Plethodon glutinosus* was analyzed electrophoretically in 135 samples from populations collected

throughout its range. It was found that *P. glutinosus* is a complex comprised of at least 16 genetically divergent species and semispecies, most of which probably began differentiating during the late Miocene or Pliocene. One form, *P. teyahalee*, is probably of hybrid origin.

The most different form, *P. kentucki* Mittleman, occurs in sympatry throughout the Cumberland Plateau with the northern, large, brassy-spotted species here recognized as *P. glutinosus* (Green). Two other highly differentiated species, *P. teyahalee* Hairston and *P. aureolus* Highton, are sympatric throughout the range of *P. aureolus* in southeastern Tennessee and adjacent North Carolina. Both *P. teyahalee* and *P. aureolus* are known to be sympatric with *P. glutinosus* at a single locality in Polk County, Tennessee, without evidence of hybridization (Highton, 1984), but their ranges are otherwise parapatric to that of *P. glutinosus*. All forms except *P. kentucki* and *P. aureolus* replace each other geographically and are considered semispecies. Names are available for some of the species, but new names are provided for seven undescribed taxa. The problems associated with recognizing forms taxonomically that are primarily distinguished on the basis of allozyme data are discussed.

The northernmost populations of *P. chlorobryonis*, *P. glutinosus*, and *P. cylindraceus* have much less geographic genetic variation than the more southern populations of most of the forms (an exception is the genetic similarity of the southern populations of *P. mississippi*). The genetic similarity of northern populations is probably due to the recent northward expansion of their ranges after the withdrawal of the Wisconsin glaciation during the last 15,000 years.

Mean heterozygosity in populations varies from a high of 0.23 in two populations of *P. chattahoochee*, the most variable species, to a low of 0 in *P. savannah* and one population each of *P. chlorobryonis* and *P. kisatchie*. The overall mean heterozygosity of all 135 populations of the *P. glutinosus* complex is 0.06. Of the loci evaluated electrophoretically, 21 of 22 (95%) have detectable genetic variation. Differing rates of evolution are apparent at the loci evaluated. At some loci it is possible to suggest the ancestral genotype.

The maximum size of coastal plain salamanders of all species is usually below 74 mm and that of noncoastal plain populations is usually above 74 mm. Exceptions are the small-sized species *P. aureolus* and *P. kentucki*.

Addendum

Since this paper was prepared, it was discovered that a mistake in coding one locus in population 116 resulted in an error in its placement on the phenogram in Figure 1. When corrected, this, the only population of light-chinned Texas *P. albagula,* has an average Nei genetic distance to other populations of that species of 0.16, making it the most divergent population of *P. albagula.* Its relationship to other Texas populations is obviously in need of further study (see p. 19).

Part II
Immunological Analysis of Geographic Variation in Plasma Albumins

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and Richard Highton

ACKNOWLEDGMENTS

This study was in partial fulfillment of the requirements for the doctoral degree in the Department of Genetics and Development at the University of Illinois at Urbana-Champaign (GCM). Portions of this study were supported by the National Science Foundation (DEB 82-01587 & BSR 83-19969 to LRM and DEB-76-10583 & DEB-79-03858 to RH) and the Department of Genetics and Development at the University of Illinois. We thank Jerry D. Coyne, Allan Larson, and David B. Wake, for helpful suggestions on the manuscript.

INTRODUCTION

The quantitative immunological technique of micro-complement fixation (MC'F) has been used extensively in studies of phylogenetic relationships among diverse amphibians (for example, Maxson *et al.*, 1979; Maxson, 1984; Maxson and Roberts, 1985; Hutchinson and Maxson, 1987). Micro-complement fixation studies of serum albumin (a 580 amino acid protein) demonstrate that MC'F offers a sufficiently rapid and accurate means of estimating sequence differentiation (Wilson *et al.*, 1977; Maxson and Maxson, 1986) to allow good phylogenetic inference over a long range of time (approximately 100 million years). Protein sequence information has a known genetic foundation and consequently can be analyzed phylogenetically and independently of any hypotheses about evolutionary rates. When evolutionary rates are unequal (and they generally do exhibit some variance), the resultant phylogenetic tree will display this fact of nonequal rates without any bias.

Phylogenetic trees derived from molecular data do not group species together just because they are similar morphologically. Serum albumin, which evolves fairly rapidly, provides a data base such that the number of events between speciation nodes is large. Thus, each internodal lineage in a tree typically is defined by numerous events (generally ten or more). The number of detected changes along each of the lineages is a direct measure of the confidence in inferring that lineage.

Micro-complement fixation analysis of proteins of known sequence has provided evidence that the immunological distance (ID) measured is a good estimate of the amino acid sequence difference between the two proteins compared (Champion *et al.*, 1975; Wilson *et al.*, 1977; Prager *et al.*, 1978; Benjamin *et al.*, 1984; Maxson and Maxson, 1986). For albumin, it is estimated that each ten ID units represents roughly ten amino acid substitutions in the albumins being compared (Maxson and Wilson, 1974; Maxson and Maxson, 1986). Using MC'F analyses of albumin evolution, single amino acid substitutions can be detected and comparisons between homologous albumins can be made until they

differ in their total sequence by 35–40% (Maxson and Maxson, 1979, 1986).

Because molecular evolution proceeds independently of morphological evolution (Wilson, 1985; Beverley and Wilson, 1982; Maxson *et al.*, 1979; Wilson *et al.*, 1974), MC'F studies have uncovered cryptic species (Maxson *et al.*, 1977; Maxson, 1981; Maha *et al.*, 1983) and detected cases of convergent morphological evolution (Maxson and Wilson, 1974; Maxson, 1977; Maxson *et al.*, 1982). Recent work has shown MC'F capable of identifying interspecific hybrids (Maxson *et al.*, 1987). Thus, the application of MC'F as a sensitive probe of protein sequence evolution has addressed many interesting questions in amphibian phylogeny and biogeography (for example Maxson and Roberts, 1985; Maxson *et al.*, 1984; Maxson and Wake, 1981).

Most of these earlier studies were concerned primarily with interspecific rather than interpopulation comparisons. Several workers have shown that gel electrophoresis of many proteins, not MC'F, is the molecular technique of greatest sensitivity for studies of intraspecific protein variation (Bush and Kitto, 1978; Maxson and Maxson, 1979; Prager and Wilson, 1980). However, MC'F has been shown to be a sensitive detector of single amino acid differences between orthologous proteins (Cocks and Wilson, 1969; Maxson and Maxson, 1986). Indeed, some MC'F studies have compared allopatric populations of the same species and demonstrated some degree of albumin polymorphism between these populations (Maxson and Wilson, 1974; Maxson, 1981; Maha *et al.*, 1983).

While studying the phylogenetic relationships among the then recognized 26 species of *Plethodon* (Maxson *et al.*, 1979), we prepared an antibody to albumin from a population of *P. glutinosus* from Ulster County, New York. Highton (1970, 1972) had shown that there were several locally differentiated populations of the *P. glutinosus* complex in the southern Appalachian Mountains and in the middle Atlantic states. Accordingly, we tested individuals from several populations that Highton had designated as "brassy-spotted," "white-spotted" or "Coastal Plain" types. Our preliminary survey demonstrated that MC'F comparisons could distinguish *P. glutinosus* albumin alleles. The realization that we could readily measure albumin differentiation among these groups prompted us to begin a more extensive, simultaneous immunological and electrophoretic study of the *P. glutinosus* complex from throughout its range.

MATERIALS AND METHODS

Antisera to purified albumin of *P. glutinosus* from Ulster County, New York, was available from the earlier study of *Plethodon* (Maxson *et al.*, 1979). Additional antisera were prepared to the purified albumins of *P. chlorobryonis* from McCormick County, South Carolina, *P. albagula* from Garland County, Arkansas, and *P. jordani* from Knob Mountain, Tazewell County, Virginia (reported in Maha *et al.*, 1983). The albumins used to raise antisera were obtained from salamanders known to be homozygous for alleles *b* (*P. chlorobryonis*), *d* (*P. glutinosus*), and *e* (*P. albagula*) from populations 21, 70, and 126, respectively (Appendix 1). All antisera were produced in New Zealand White rabbits over a 13 week period according to procedures outlined by Maxson *et al.*, (1979). All antisera were judged to be directed solely to serum albumin as evidenced by a single precipitin arc in immunoelectrophoresis when tested with salamander plasma. Additionally, results of MC'F tests with purified albumin and whole plasma were indistinguishable.

Plasma from individual salamanders from 135 populations of the *P. glutinosus* complex was compared in MC'F tests with the three antisera according to procedures described by Champion *et al.*, (1974). For populations where gel electrophoresis had detected albumin polymorphism, only individuals homozygous for each allele were used in MC'F tests. Heterozygotes were not used in this study. When the same individual salamander was compared to an antiserum several times, the immunological distance (ID) measured was within ± 2 units of an average ID. This experimental variation in ID is the same order of magnitude found in previous studies and reflects the lower limit of resolution of the MC'F methodology (Maxson and Maxson, 1979, 1986).

The 135 populations studied are the same as described in Part 1 and voucher specimens from most localities will be deposited in the collection of the National Museum of Natural History. Nei distance (Nei, 1972) estimates of genetic differentiation are from Part 1. The phylogenetic tree in Figure 18 was constructed using a modification (Maxson *et al.*, 1979) of the distance Wagner procedure (Farris, 1972).

RESULTS AND DISCUSSION

The 21 hour titer for the four antisera varied from 2,000 to 4,100, with an average of 3,000. The slope of all four antisera was 400. Both

parameters are typical of titers and slopes reported in other amphibian studies (Maxson *et al.*, 1979; Maxson, 1984).

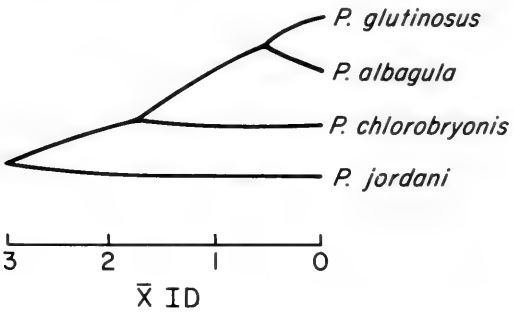

Fig. 18. Phylogeny depicting branching relationships among species of the *Plethodon glutinosus* complex for which albumin antisera were available, and *P. jordani*. Details of tree construction are in the text. Average immunological distances between branches of tree are indicated.

Table. 7. Immunological comparisons (given in immunological distance units) of three samples of the *Plethodon glutinosus* complex and one sample of *P. jordani* (from Maha, *et al.*, 1983).

	Antisera			
	P. glutinosus complex			*P. jordani*
Antigen	NY	AR	SC	VA
P. glutinosus complex				
NY (*P. glutinosus*)	0	1	4	5
AR (*P. albagula*)	1	0	6	6
SC (*P. chlorobryonis*)	3	2	0	4
P. jordani				
VA	5	9	8	0

The reciprocal data from comparisons of the four antisera are presented in Table 7. The average deviation from perfect reciprocity is 2 units. The percent standard deviation from reciprocity (Maxson and Wilson, 1975) normally calculated for MC'F studies is 26.5 percent. This value is atypically high compared to earlier studies where these values typically average 10–15 percent. The high 26.5 percent is an artifact of the low ID values measured between these *Plethodon* popula-

tions. A similar situation was reported in a study of albumin evolution in populations of marsupial tree frogs (Scanlan *et al.*, 1980). Because the average sensitivity of immunological distance measurements is ±2 ID (Maxson and Maxson, 1979), the nonreciprocity of this matrix becomes trivial.

Average IDs were computed from the reciprocal values presented in Table 7. These values were used to draw the tree shown in Figure 18. The goodness-of-fit statistics for the tree are: standard deviation (Fitch and Margoliash, 1967) = 10.1 percent; f (Farris, 1972) = 2.0 percent; F (Prager and Wilson, 1976) = 7.4 percent; cophenetic correlation coefficient (Sokal and Rohlf, 1962) = 0.959. All of these parameters indicate a reasonable fit of the data to the tree. For comparison, typical Fitch-Margoliash standard deviations in amphibian studies average 15 percent (Maxson, 1981).

Inspection of Table 7 indicates that the *P. glutinosus* and *P. albagula* populations have the most similar albumins (1 ID), and that *P. jordani* has an albumin most differentiated from the three other antisera (an average ID of 6). *P. jordani* was initially included to serve as an outgroup for analysis of relationships within the *P. glutinosus* complex. Although it served to root the cladogram in Figure 18, Maha *et al.* (1983) showed that the albumin of *P. jordani* is usually more similar to the albumins of some members of the *P. glutinosus* complex than it is to the albumins of *P. kentucki*. Because *P. jordani* could no longer serve as an outgroup to the entire *P. glutinosus* complex, no further comparisons were made with this antiserum.

Figure 18 may be somewhat misleading if we consider more extensive one-way albumin comparisons between the three alleles used to prepare antisera (summarized in Table 8). Using antisera to *P. glutinosus* allele *d*, the average ID to 13 samples of *P. albagula* allele *e* is 2.6 ±0.4, whereas the *P. albagula* antisera run with 36 samples of *P. glutinosus* allele *d* gives an average ID of 1.6 ±0.2. The grand mean is 1.9 ±0.2, in contrast to the value of 1 obtained between the two populations used to produce the antisera. Similar comparisons of the *P. chlorobryonis* antisera to the 36 samples of *P. glutinosus* allele *d* yield an average ID of 4.3 ±0.4, agreeing with the single population comparison of 4. Another alternative that cannot be ruled out is that all three species are roughly equidistant from one another and would arise at a trifurcation almost immediately after the lineage to *P. jordani* diverged. It is also possible that all four lineages are roughly equally divergent from one another.

Table 8. Summary of immunological distances to three antibodies of the *P. glutinosus* complex.

Species	Allele	N	New York antibody (d)		Arkansas antibody (e)		South Carolina antibody (b)	
			Range	$\bar{x} \pm$ S.E.	Range	$\bar{x} \pm$ S.E.	Range	$\bar{x} \pm$ S.E.
P. kentucki	a	2	7–8	7.5 ±0.5	7–9	8.0 ±1.0	8	8.0 —
P. kentucki	b	2	13	13.0 —	13	13.0 —	12–13	12.5±0.5
P. teyahalee	c	6	1–4	2.8 ±0.5	1–7	3.8 ±0.8	4–10	7.0±1.0
P. glutinosus	c	3	3–5	4.0 ±0.6	3–5	4.0 ±0.6	3–9	6.0±1.7
P. kisatchie	c	2	1–4	2.5 ±1.5	1–3	2.0 ±1.0	3–7	5.0±2.0
P. chattahoochee	d	4	3–6	4.5 ±0.6	3–4	3.5 ±0.3	2–5	3.8±0.6
P. chlorobryonis	d	12	0–9	2.9 ±0.7	0–9	3.2 ±0.7	0–7	2.0±0.6
P. variolatus	d	7	1–7	3.1 ±0.8	2–5	2.7 ±0.4	0–6	3.0±0.8
P. glutinosus	d	36	0–4	0.4[1] ±0.2	0–4	1.6 ±0.2	0–10	4.3±0.4
P. mississippi	d	11	0–5	2.7 ±0.5	1–3	2.3 ±0.2	4–11	8.5±0.7
P. cylindraceus	d	3	1–3	2.3 ±0.7	2–3	2.7 ±0.3	7–10	8.0±1.0
P. kiamichi	e	2	2–4	3.0 ±1.0	0–2	1.0 ±1.0	6–8	7.0±1.0
P. grobmani	e	10	2–5	3.5 ±0.3	0–3	1.2 ±0.3	4–12	7.3±0.8
P. cylindraceus	e	9	0–6	2.3 ±0.7	0–6	3.0 ±0.6	4–13	8.1±0.9
P. albagula[2]	e	13	0–5	2.6 ±0.4	0–2	0.3[1] ±0.2	4–14	8.1±0.8
P. aureolus	e	2	2	2.0 —	0–2	1.0 ±0.7	1–2	1.5±0.5
P. ocmulgee	h	4	6–7	6.5 ±0.3	3–5	4.5 ±0.5	6–11	8.3±1.1

[1] Sample size one less because homologous comparison omitted.
[2] Includes samples 92 and 116–127.

The data presented in Table 7 show that the *P. chlorobryonis* antibody usually gives higher ID values in reciprocal tests. The data from comparisons of all populations to the three antisera are given in Appendix 3. Table 9 presents average IDs for individual alleles within each species. The same pattern of higher values with *P. chlorobryonis* is observed. Thus, ID values involving comparisons to this antibody may be slightly inflated.

Individual salamanders from 135 populations of slimy salamanders were compared to our reference panel of three antisera (Appendix 3). In populations where there were multiple albumin alleles, only individuals homozygous for each different allele were sampled. The IDs for all individuals tested are summarized in Table 9, along with the designations of the electrophoretically identified alleles. When this study was initiated, all populations were considered a single species, *P. glutinosus*. Based on the analysis in Part 1, 16 species are now recognized.

Table 9. Average immunological distances of 135 populations of the *P. glutinosus* complex to reference antisera.

(alleles)	*P. glutinosus* (d)	*P. albagula* (e)	*P. chlorobryonis* (b)
P. teyahalee			
N = 6 (c)	2.8	3.8	7.0
P. chattahoochee			
N = 4 (d)	4.5	3.5	3.8
N = 1 (e)	6.0	4.0	2.0
P. chlorobryonis			
N = 1 (b)	3.0	2.0	0.0
N = 12 (d)	2.9	3.2	2.0
N = 1 (g)	9.0	10.0	12.0
P. variolatus			
N = 7 (d)	3.1	2.7	3.0
P. ocmulgee			
N = 1 (d)	3.0	0.0	7.0
N = 4 (h)	6.5	4.5	8.3
P. glutinosus			
N = 1 (b)	5.0	4.0	4.0
N = 3 (c)	4.0	4.0	6.0
N = 36 (d)	0.4	1.6	4.3
N = 1 (e)	1.0	1.0	4.0
N = 2 (h)	7.0	5.0	8.5
P. kiamichi			
N = 2 (e)	3.0	1.0	7.0
P. mississippi			
N = 11 (d)	2.7	2.3	8.5
P. kisatchie			
N = 2 (c)	2.5	2.0	5.0
N = 1 (d)	5.0	3.0	7.0
P. sequoyah			
N = 1 (d)	2.0	3.0	14.0

(alleles)	*P. glutinosus* (d)	*P. albagula* (e)	*P. chlorobryonis* (b)
P. grobmani			
N = 10 (e)	3.5	1.2	7.3
N = 1 (g)	7.0	6.0	8.0
P. cylindraceus			
N = 1 (c)	4.0	5.0	10.0
N = 3 (d)	2.3	2.7	8.0
N = 9 (e)	2.3	3.0	8.1
N = 1 (h)	7.0	6.0	11.0
P. albagula			
N = 1 (c)	5.0	0.0	6.0
N = 13 (e)	2.6	0.3	8.1
N = 1 (i)	4.0	5.0	13.0
P. savannah			
N = 1 (h)	7.0	4.0	5.0
P. aureolus			
N = 1 (d)	3.0	2.0	0.0
N = 2 (e)	2.0	1.0	1.5
P. kentucki			
N = 2 (a)	7.5	8.0	8.0
N = 2 (b)	13.0	13.0	12.5

The highest immunological distances are found in the comparisons of *P. kentucki* allele *b* with all three antibodies. The other common *P. kentucki* allele *a* is also among the most different from the panel of antibodies. This result agrees with the finding that *P. kentucki* is genetically the most divergent species of the *P. glutinosus* complex (Part 1).

The *P. chlorobryonis* antibody was prepared from animals homozygous for allele *b*. This allele is absent in most other populations of *P. chlorobryonis*, but its mean ID to the common allele *d* of *P. chlorobryonis* (2.0) is lower than to most other alleles in other species. With the exception of *P. aureolus* (discussed below), only the comparisons to allele *d* in *P. chattahoochee* and *P. variolatus* are less than 4.0. The latter two species are genetically most closely related to *P. chlorobryonis* (Part 1).

Throughout its range, *P. kentucki* is sympatric with *P. glutinosus*. Despite the difficulty in distinguishing the two species using morphological criteria, *P. kentucki* has two common albumin alleles that are

distinct from that of *P. glutinosus* by both electrophoretic and immunological criteria (Maha *et al.*, 1983, Highton and MacGregor, 1983). Throughout its range, *P. aureolus* is sympatric with *P. teyahalee* and the two species are sympatric with *P. glutinosus* at one locality in Polk County, Tennessee (Highton, 1984). All other species replace each other geographically (Part 1).

Surprisingly, the immunological distances of the albumins of all three populations of *P. aureolus* to the panel of antibodies are low (0–3 ID). This result is unexpected in light of the considerable amount of electrophoretic genetic differentiation of this species compared to all the others (Part 1). Perhaps the *P. aureolus* albumin incurred significantly fewer substitutions than some of the other loci screened electrophoretically.

Tables 8 and 9 show considerable albumin variation within several of the species of the *P. glutinosus* complex. Within species, there are alleles that differ (by immunological criteria) from those of the reference antisera. For example, among the 40 populations of *P. glutinosus*, five albumin electromorphs are observed. Alleles designated *b* and *c* have essentially the same pattern of immunological cross-reactivity as do alleles *d* and *e*. Allele *h* exhibits still a third pattern. We interpret this amount of differentiation as indicative of a considerable amount of evolutionary divergence. Such albumin differentiation has also been found in other amphibians with little or no discernable morphological variation (Maxson *et al.*, 1977; Maxson, 1981; Maxson, unpub.).

Twenty-four of the 29 populations of *P. glutinosus* from the northern portion of its range (NY, NJ, PA, MD, WV, VA, OH, IN, IL, and KY) have immunological distances of 0 to the homologous New York antibody. These northern populations are all genetically closely related. This species very likely extended its range northward from southeastern Kentucky since the last glaciation (Part 1). These results might be expected if the source populations were monomorphic for a single albumin. The remaining populations of *P. glutinosus* with the *b*, *c*, *e*, and *h* alleles occur in the southern part of the range of the species (Figs. 1 & 11). The Nei genetic distances (Appendix 3) between the New York population and these southern populations are higher ($\bar{D} = 0.09$) than the Nei genetic distances to the northern populations ($\bar{D} = 0.03$).

Another striking feature of these data is that alleles with the same electrophoretic mobility (same letter designation) do not always behave identically to our panel of antisera. For example, allele *d* from New York

P. glutinosus is immunologically different from allele *d* found in *P. chattahoochee*, *P. chlorobryonis*, *P. variolatus*, *P. ocmulgee*, *P. mississippi*, *P. kisatchie*, *P. sequoyah*, *P. cylindraceus*, and *P. aureolus*. Without additional antisera to these other species, it is not possible to determine if all *d* alleles are identical to one another. However, by examining their pattern of cross reactivity to the other two antisera (compare the *d* allele of *P. chattahoochee*, *P. chlorobryonis*, and *P. variolatus* with the *d* allele of *P. mississippi* and *P. cylindraceus*, Tables 8 & 9) it appears that there are at least two additional "*d*" alleles. These results are not surprising in light of the genetic distances among these species.

Another example involves allele *e* for which the antiserum to *P. albagula* was prepared. The *e* alleles of *P. grobmani* and *P. kiamichi* show a common pattern of cross reactivity to *P. albagula*. However, allele *e* in *P. aureolus* shows yet another pattern of cross reactivity.

It would be expected that some mutations might not change electrophoretic mobility (Reichlin *et al.*, 1966; Cocks and Wilson, 1969; Prager and Wilson, 1976; King and Wilson, 1975). Sequential gel electrophoresis (Coyne, 1982) might also have detected some of these additional alleles. Maiorana (unpublished data) found that the mobility of allele *d* in *P. glutinosus* was different from the *d* allele in *P. chlorobryonis* when compared using the method of Smithies (1959). King and Wilson (1975) estimated that only 28 percent of all amino acid substitutions that occur are resolved by standard gel electrophoresis but Coyne (1982) suggests a higher estimate (about 50 percent). The fact that banding patterns seen on electrophoretic gels are phenotypes, and thus do not necessarily reflect the complete underlying genotype, has been discussed by others (Allendorf, 1977; King and Ohta, 1975). It has been demonstrated, however, that MC'F can detect those substitutions that do not change the overall net charge of the protein (Cocks and Wilson, 1969; Ibrahimi *et al.*, 1980). Our results also show the ability of MC'F to detect additional alleles.

Not all alleles show this confusing pattern. For example, allele *h* in *P. ocmulgee* (populations 31–34), *P. glutinosus* (populations 72, 73), *P. cylindraceus* (population 111), and *P. savannah* (population 128) all show similar patterns of cross reactivity to the panel of antisera. It would be interesting to find out whether allele *h* is the same in these genetically rather different but geographically parapatric species.

Some alleles may not be distinguished by immunological analysis. For example, MC'F did not distinguish between alleles *d* and *e* in *P. glutinosus*

or *d* and *e* of *P. cylindraceus* (Tables 8 & 9). This inability to discriminate between alleles is not surprising. An antiserum to each allele in the study as well as many more populations would probably be needed to discriminate between all of the different albumins.

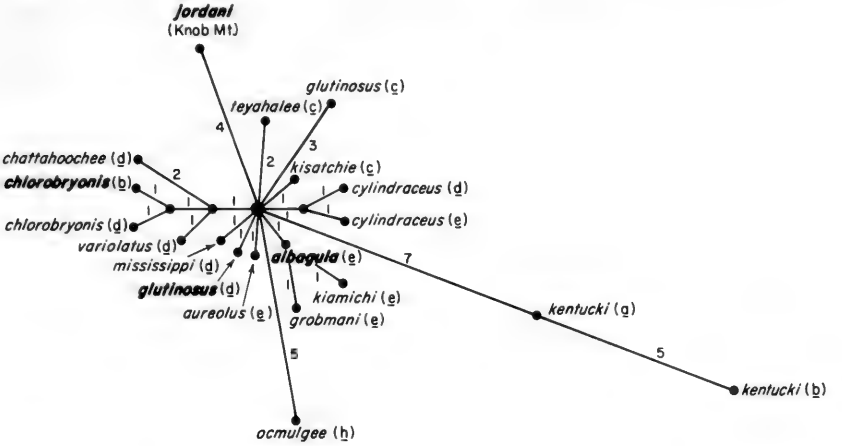

Fig. 19. Network of inferred relationships among species in the *P. glutinosus* complex, indicating all of the common alleles detected by gel electrophoresis. The distances along the branches are immunological distance units.

An analysis of the correlation of immunological distance and Nei genetic distance was performed because there are 431 comparisons. The correlation coefficient was 0.585, considerably lower than that observed in earlier studies where correlations of between 0.8 and 0.9 were reported (Maxson and Maxson, 1979; Highton and Larson, 1979; Wyles and Gorman, 1980). However, all earlier studies involved IDs ranging from 0–80 ID, with very few IDs less than 10 units. This study, on the other hand, involved IDs all less than 14. Despite the small range of immunological distance in these comparisons, the correlation was still significant at the 0.01 level. The slope of the linear regression line for these data is 15.5, also somewhat lower than that in previous studies of salamanders: *Hydromantes* (28), *Pseudoeurycea* (23), and *Plethodon* (24) (Maxson and Maxson, 1979). This variation in slope should serve as a cautionary warning to investigators who use Nei genetic distances to estimate time of divergence from albumin immunological studies. The apparent variation in the slope appears to be attributable to the species studied as well as to the suite of loci compared.

Figure 19 is a hypothetical network showing possible relationships

among the more common albumin alleles. This network is reasonably consistent with the MC'F data. Because we had antibodies to only three alleles, many of the hypothetical relationships are tentative. In general, the relationships agree with those described in Part 1. Examples are the similarity of *P. chattahoochee*, *P. chlorobryonis*, and *P. variolatus*, the isolated position of *P. kentucki*, and the approximately equidistant relationship of the remaining species. Amino acid sequencing of the various albumins of the *P. glutinosus* complex would be needed to better understand the evolutionary relationships of these proteins.

SUMMARY

This immunological analysis of albumin differentiation in the *P. glutinosus* complex has shown that quantitative MC'F can detect a considerable amount of the variation present in closely related species. As suggested earlier (Maxson and Maxson, 1979) electrophoretic analysis is the methodology of choice for studying closely related populations. Although MC'F can detect single amino acid substitutions, even in the absence of a charge change, an antiserum to each genetically differentiated population is needed for more definitive work. With the three antisera used in this study, we could identify populations as different but could not characterize taxonomic groupings as is possible with electrophoretic analysis (Part 1). By combining MC'F and electrophoretic analyses of salamander albumins, we have demonstrated more underlying genetic variation than is resolvable by either technique alone.

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Appendix 1. List of localities

Locality		Population	Latitude			Longitude			Elevation
State	County or Parish	No.	°	′	″	°	′	″	m
Alabama	Barbour	100	31	42	17	85	40	13	113
	Butler	97	31	34	22	86	44	12	91
	Clarke	87	31	32	55	87	55	48	30
	Cleburne	46	33	29	15	85	47	28	378
	Etowah	36	34	02	51	86	10	38	274
	Lawrence	78	34	18	25	87	20	10	262
	Macon	73	32	29	30	85	36	08	91
	Tuscaloosa	77	33	03	54	87	43	35	91
Arkansas	Dallas	127	33	54	18	92	34	02	119
	Garland	126	34	32	32	93	01	42	207
	Montgomery	92	34	39	20	93	57	00	588
	Pope	117	35	38	28	93	04	03	549
	Stone	121	35	59	05	92	16	02	311
	Union	88	33	17	58	92	31	44	34
Florida	Columbia	94	29	54	45	82	35	02	17
	Hillsborough	93	27	51	53	82	13	00	15
	Jackson	102	30	49	25	85	18	15	40
	Lake	96	29	04	54	81	34	45	5
	Leon	98	30	25	48	84	31	45	27
Georgia	Bacon	30	31	26	31	82	25	53	49
	Bulloch	31	32	23	03	81	49	59	55
	Chatham	28	32	08	42	81	09	18	3
	Effingham	29	32	34	02	81	20	52	30
	Fannin	8	34	52	52	84	33	57	1,061
	Habersham	20	34	37	24	83	29	27	418
	Henry	72	33	29	53	84	11	03	232
	Lanier	95	31	02	33	83	05	54	58
	Long	34	31	42	36	81	45	16	20
	Rabun	10	34	55	48	83	32	46	607
	Richmond	128	33	19	48	82	03	49	101
	Schley	99	32	10	26	84	22	17	137

Locality		Population	Latitude			Longitude			Elevation
State	County or Parish	No.	°	′	″	°	′	″	m
	Terrell	101	31	39	44	84	27	54	85
	Towns	9	34	52	21	83	48	31	1,353
	Union	7	34	39	10	84	08	20	981
	Upson	33	32	47	38	84	15	30	137
	Wheeler	32	32	05	38	82	53	35	49
Illinois	Pope	42	37	22	54	88	40	20	137
	Union	41	37	32	43	89	26	14	134
Indiana	Crawford	40	38	16	35	86	32	10	198
	Jackson	52	38	50	44	86	01	25	229
	Parke	44	39	53	14	87	11	20	174
Kentucky	Harlan	66,135	36	56	01	83	11	51	793
	Harlan	53,133	36	55	03	82	54	04	1,238
	McCreary	47	36	52	12	84	21	55	299
	Muhlenberg	39	37	17	03	87	08	27	137
	Pike	63,134	37	18	23	82	35	50	305
	Scott	68	38	23	23	84	34	02	250
	Warren	43	37	01	22	86	18	51	162
Louisiana	Grant	90	31	43	15	92	28	02	30
	Washington	85	30	52	00	90	00	08	73
	Winn	89	31	52	12	92	33	30	30
Maryland	Frederick	55	39	37	50	77	28	17	381
Mississipi	Forrest	86	30	55	40	89	10	36	88
	Lowndes	84	33	29	25	88	20	52	58
	Scott	83	32	24	37	89	29	02	125
	Tishomingo	79	34	36	38	88	11	56	177
	Winston	82	33	12	35	88	59	17	140
Missouri	Iron	118	37	33	37	90	40	16	381
	Miller	120	38	04	41	92	31	15	232
	Wayne	119	36	56	33	90	15	09	128
New Jersey	Union	48	40	40	42	74	23	10	91
New York	Tompkins	59	42	19	55	76	39	34	396
	Ulster	70	41	55	44	74	06	10	152

Locality		Population	Latitude			Longitude			Elevation
State	County or Parish	No.	°	′	″	°	′	″	m
North Carolina	Burke	111	35	41	43	81	43	02	347
	Columbus	13	34	19	55	78	52	36	26
	Craven	14	35	17	19	77	07	39	8
	Graham	5	35	23	33	83	46	26	1,116
	Halifax	15	36	12	48	77	34	38	24
	Haywood	4	35	44	44	83	04	43	692
	Henderson	115	35	10	20	82	26	10	829
	Macon	3	35	06	20	83	17	05	1,036
	Madison	6	35	48	50	82	56	58	1,207
	Madison-Unicoi (Tn)	113	36	00	30	82	36	32	988
	Mitchell-Unicoi (Tn)	114	36	06	36	82	21	40	1,036
	Montgomery	103	35	13	18	79	47	25	168
	Pender	19	34	31	17	77	48	38	8
	Tyrrell	18	35	48	38	76	05	17	1
Ohio	Brown	54	38	43	55	83	47	10	213
	Meigs	50	39	02	58	81	58	45	232
	Wayne	67	40	46	50	81	54	50	299
Oklahoma	Adair	125	35	50	13	94	39	20	320
	Le Flore	76	34	36	55	94	29	50	640
	Le Flore	75	34	37	40	94	48	43	716
	McCurtain	91	34	07	29	94	40	15	140
	Sequoyah	124	35	37	47	94	34	50	195
Pennsylvania	Beaver	60	40	29	50	80	25	44	317
	Bedford	69	39	47	24	78	39	40	366
	Cambria	56	40	42	07	78	48	08	451
	Cameron	62	41	28	43	78	11	27	427
	Snyder	61	40	43	09	76	59	48	503
	Susquehanna	58	41	50	06	76	02	15	351
South Carolina	Aiken	22	33	25	32	81	52	48	79
	Allendale	25	33	01	30	81	15	00	41
	Berkeley	27	33	08	00	79	47	06	6
	Charleston-Dorchester	23	32	53	44	80	08	12	9
	Chester	112	34	44	32	81	05	36	137
	Chesterfield	11	34	43	55	80	02	57	55
	Florence	12	33	54	10	79	26	25	9

Locality		Population	Latitude			Longitude			Elevation
State	County or Parish	No.	°	′	″	°	′	″	m
	Jasper	26	32	33	10	81	10	10	18
	Jasper	24	32	36	14	80	54	08	6
	McCormick	21	34	01	47	82	23	55	149
Tennessee	Bledsoe	37	35	38	32	85	19	55	506
	Blount	45	35	38	20	83	44	52	549
	Cocke	74	35	54	07	83	17	04	1,009
	Henderson	80	35	48	08	88	15	36	165
	Monroe	1,130	35	27	45	84	01	37	872
	Monroe	2,129	35	21	20	84	04	42	1,128
	Montgomery	71	36	31	00	87	30	35	152
	Moore	35	35	20	55	86	20	28	299
	Polk	38	35	09	15	84	36	27	604
	Polk	131	35	11	33	84	29	43	299
	Shelby	81	35	15	18	89	45	10	98
Texas	Hays	116	29	56	27	97	54	14	198
	Kerr	123	29	55	00	99	11	48	573
	Travis	122	30	18	17	97	46	16	158
Virginia	Augusta	108	38	12	20	79	15	52	439
	Bedford	106	37	29	27	79	33	22	957
	Botetourt	107	37	39	33	79	41	10	878
	Dinwiddie-Sussex	17	36	56	34	77	29	43	40
	Fairfax	105	38	45	14	77	17	53	91
	Giles	49	37	14	56	80	51	48	1,143
	Grayson-Wythe	104	36	45	47	81	13	22	1,134
	Pittsylvania	110	36	34	13	79	26	06	152
	Russell	51	36	56	55	81	52	33	1,067
	Southampton	16	36	52	26	76	57	28	18
	Wise	65,132	36	53	42	82	37	58	1,165
West Virginia	Clay	64	38	21	27	81	07	48	427
	Hampshire	109	39	17	08	78	23	58	335
	Preston	57	39	27	24	79	31	08	792

Appendix 2. Genic variation and heterozygosity estimates in samples of the *Plethodon glutinosus* complex.

	P. teyahalee						*P. chattahoochee*			
Population No.	1	2	3	4	5	6	7	8	9	10
n:	39	32	25	30	35	30	30	30	30	30
Locus										
Alb	c (n=48)	c (n=30)	c (n=26)	c (n=35)	c(.971) e(.029)	c (n=99)	d (n=46)	d(.996) g(.004) (n=132)	d (n=171)	d(.686) e(.314) (n=35)
α-Gpd	b (n=30)	b	b	b	b	b	b(.724) e(.276) (n=29)	b(.983) e(.017)	b(.850) e(.150)	b(.983) e(.017) (n=29)
Est	e	c(.031) e(.875) s(.094)	e	e(.950) n(.050)	e(.952) j(.048) (n=31)	e	a(.017) c(.117) g(.083) j(.017) k(.033) l(.033) n(.033) p(.050) r(.050) t(.267) x(.067) z(.017)	a(.017) b(.017) c(.033) d(.033) e(.050) h(.017) j(.017) l(.100) m(.050) p(.400) t(.217) y(.017)	a(.017) c(.017) d(.017) e(.050) g(.050) h(.017) j(.100) k(.050) p(.283) r(.017) t(.150) w(.017)	c(.019) e(.167) g(.019) h(.019) j(.056) k(.037) n(.204) p(.241) q(.019) r(.019) s(.019) t(.074)

	P. teyahalee						*P. chattahoochee*			
Population No.	1	2	3	4	5	6	7	8	9	10
n:	39	32	25	30	35	30	30	30	30	30
Locus										
							aa(.033) bb(.017) ee(.033) ff(.017) hh(.017) ii(.100)	z(.033)	y(.033) z(.117) dd(.033) ee(.017) ff(.017)	v(.019) y(.037) z(.037) ee(.019) (n=27)
Fum	b	b	b (n=24)	b	b	b	b(.750) c(.250)	b(.933) c(.067)	b	a(.017) b(.983) (n=29)
Gdh	b(.714) c(.286) (n=35)	b	b	b	b	b	b	b	b	b
Got-1	h	h(.984) j(.016)	h	h(.933) j(.067)	h(.971) j(.029)	h	h(.033) j(.967)	h(.100) j(.900)	h(.117) j(.883)	h(.167) j(.717) k(.117)
Got-2	e	e	e	e	e	e	e	e	e	a(.033) e(.967)
Idh-1	b(.897)	b(.828)	b(.960)	b	b(.971)	b	b(.517)	b(.150)	b(.533)	b(.150)

	e(.103)	e(.172)	e(.040)		e(.029)		c(.150) e(.333)	c(.033) e(.817)	c(.133) e(.333)	c(.333) e(.517)
Idh-2	c	c(.969) d(.031)	c	c(.883) e(.117)	c(.956) d(.044) (n = 34)	c(.983) e(.017)	c(.967) e(.033)	c(.964) e(.036) (n = 28)	c(.950) e(.050)	c(.983) e(.017)
Ldh (heart)	f	f	f	f	f	f	b(.017) f(.933) h(.050)	d(.033) f(.967)	d(.050) f(.917) h(.033)	d(.033) f(.917) h(.050)
Ldh (muscle)	g(.355) h(.645) (n = 38)	d(.016) h(.984)	h (n = 24)	h	h	h	g(.900) h(.100)	g(.883) h(.117)	g(.050) h(.950)	h (n = 29)
Lap	a (n = 36)	a(.969) c(.016) d(.016)	a	a	a	a	a(.317) c(.683)	a	a(.190) c(.810) (n = 29)	a(.600) c(.400)
Mdh-1	b	b	b	b	b	b	b (n = 29)	b	b	b
Mdh-2	d (n = 38)	d	d	d	d	d	d	d	d	d(.883) f(.117)
Pep	d	d	d	d	d	d	a(.033) c(.683) d(.283)	c	c	c

	P. teyahalee						*P. chattahoochee*			
Population No.	1	2	3	4	5	6	7	8	9	10
n:	39	32	25	30	35	30	30	30	30	30
Locus										
Pgi	c(.641) g(.359)	c(.578) g(.422)	c(.780) g(.220)	c(.733) g(.267)	c(.429) g(.571)	c(.333) g(.667)	c(.467) g(.533)	c(.017) g(.983)	c(.217) g(.783)	c(.267) g(.733)
Pgm	c (n=38)	c (n=31)	c	c	c	c	c	c	c (n=29)	c
Pt-1	b	b	b (n=23)	b	b	b	b	b	b	b
Pt-2	b	b	b (n=24)	b	b	b	c	c	c	c
6-Pgd	b (n=32)	b(.891) d(.109)	a(.020) b(.960) d(.020)	b	b	b(.850) c(.033) e(.117)	b(.172) d(.569) e(.259) (n=29)	b(.517) d(.450) e(.033)	b(.086) d(.448) e(.293) f(.172) (n=29)	b(.567) d(.283) f(.150)
Trf	a(.948) b(.052) (n=67)	a(.667) b(.117) d(.083) f(.017) k(.117)	a (n=26)	a(.900) b(.029) j(.071) (n=35)	a(.986) i(.014)	a(.628) b(.054) c(.007) d(.014) e(.020)	h(.120) j(.772) n(.065) q(.043) (n=46)	d(.246) f(.042) g(.099) h(.099) j(.367)	f(.049) h(.901) k(.006) 1(.012) n(.025)	a(.014) c(.029) d(.043) f(.014) g(.071)

		(n=30)					g(.007) j(.270) (n=74)		l(.021) m(.049) o(.014) p(.063) (n=71)	p(.006) (n=81)	h(.214) k(.286) n(.300) o(.029) (n=35)
H	.073	.086	.023	.046	.036	.058	.229	.141	.171	.231		
	P. chlorobryonis											
Population No.	11	12	13	14	15	16	17	18	19	20	21	22
n:	30	30	30	29	29	32	26	30	30	30	31	25
Locus												
Alb	d(.257) g(.743) (n=101)	b(.030) d(.970) (n=33)	d (n=33)	d (n=133)	d	d (n=113)	d (n=98)	d	d	d	b(.638) d(.362) (n=156)	b(.313) d(.688) (n=24)
α-Gpd	b	b	b	b (n=28)	b	b	b	b	b	b	b (n=27)	b
Est	e(.017) k(.034) p(.034) q(.259) t(.500) v(.155) (n=29)	p(.750) t(.250)	j(.050) t(.950)	q(.018) t(.946) z(.036) (n=28)	t	t (n=31)	t	t(.817) jj(.183)	t(.484) jj(.516)	a(.050) c(.050) e(.450) g(.117) k(.117) o(.067) p(.067) z(.083)	c(.081) k(.903) p(.016)	g(.220) k(.780)

	P. chlorobryonis											
Population No.	11	12	13	14	15	16	17	18	19	20	21	22
n:	30	30	30	29	29	32	26	30	30	30	31	25
Locus												
Fum	b	b	b	b	b	b (n=27)	b	b	b (n=29)	b	b	b
Gdh	b	b	b	b	b	b	b	b	b	b	b (n=25)	b
Got-1	f(.017) h(.383) j(.600)	h(.033) j(.967)	j	h(.071) j(.929) (n=28)	j	j	h(.019) j(.981)	j	j	c(.414) h(.086) j(.500) (n=29)	h(.435) j(.565)	h(.160) j(.840)
Got-2	e	e	d(.017) e(.983)	e	e	e	e	e	d(.017) e(.983)	e	e	e
Idh-1	b(.733) e(.267)	b	b	b	b	b	b	b	b	c(.300) e(.700)	c	c
Idh-2	c	c	c	c	c (n=30)	c	c	c	c	c	c	c
Ldh (heart)	e(.017) f(.983)	f	f	f	f	f	f	f	f	d(.033) f(.967)	f	f

Ldh (muscle)	h	h	h(.983) i(.017)	h	h	h	h	h	h	h	h	h (n = 23)
Lap	a(.200) b(.800)	c	c	c	c (n = 26)	c	c	c	c	c	a(.081) c(.919)	b
Mdh-1	b	b	b	b	b (n = 25)	b	b	b	b	b	b	b
Mdh-2	d (n = 29)	d	d	d	d	d	d	d	d	d	d	d
Pep	c(.875) d(.125)	c	c	c	c	c	c	c	c	c(.783) d(.217)	c (n = 29)	c
Pgi	g	g	g	g	g	c(.016) g(.984)	g	g	g	c(.067) g(.933)	g (n = 28)	g
Pgm	c	c	c	c	c	b(.016) c(.984)	c	c	c	c(.917) d(.017) f(.067)	c	c
Pt-1	b	b	b	b (n = 28)	b (n = 28)	b	b	b	b	b	b	b (n = 23)
Pt-2	a	a	a (n = 29)	a (n = 28)	a	a (n = 27)	a	a	a	a	a (n = 18)	a (n = 22)
6-Pgd	b	b	b(.867)	b	b	b	b	b	b(.850)	b(.500)	b(.177)	b(.233)

	P. chlorobryonis											
Population No.	11	12	13	14	15	16	17	18	19	20	21	22
n:	30	30	30	29	29	32	26	30	30	30	31	25
Locus												
			d(.133)	(n=28)					d(.150)	d(.450) e(.050)	d(.565) f(.258)	d(.767) (n=15)
Trf	h(.074) o(.926) (n=101)	o (n=33)	o (n=33)	o (n=133)	o	o(.996) q(.004) (n=113)	o (n=44)	o	o	o	a(.011) g(.016) l(.005) o(.967) (n=92)	o
H	.120	.023	.018	.011	0	.003	.002	.014	.036	.135	.088	.064

	P. variolatus							*P. ocmulgee*				
Population No.	23	24	25	26	27	28	29	30	31	32	33	34
n:	30	33	30	30	33	25	25	26	33	30	28	27
Locus												
Alb	d (n=126)	d (n=128)	b(.024) d(.976) (n=41)	d (n=78)	b(.010) d(.990) (n=261)	d	d(.980) i(.020)	d (n=25)	d(.025) h(.950) k(.025) (n=60)	h (n=109)	h (n=44)	e(.074) h(.926)
α-Gpd	b	b	b (n=29)	b	b (n=27)	b	b	b	b	b	b	b(.980) d(.020) (n=25)

Est	e(.033) p(.850) t(.033) z(.083)	p	o(.117) p(.850) z(.033)	p	k(.106) o(.045) p(.273) t(.561) z(.015)	e(.440) k(.020) p(.540)	e(.120) p(.780) t(.040) z(.060)	k(.635) t(.365)	k(.076) p(.924)	c(.083) k(.717) p(.200)	k(.429) p(.571)	k
Fum	b	b	b	b	b	b	b	b	b	b	b	b (n = 26)
Gdh	b	b	b	b	b	b (n = 23)	b	a	a(.758) b(.242)	a(.767) b(.233)	a	a
Got-1	h(.135) j(.865) (n = 26)	h(.758) j(.242)	h(.583) j(.417)	h(.875) j(.125)	h(.106) j(.894)	h(.140) j(.860)	h(.180) i(.040) j(.780)	j	j	j	j	j
Got-2	e (n = 27)	e	e	e (n = 26)	e	e	e	e	e	e (n = 51)	e	e
Idh-1	e	b(.015) e(.985)	b(.017) e(.983)	b(.033) e(.967)	b(.030) e(.970)	b(.160) e(.840)	b(.420) e(.580)	b(.115) d(.885)	a(.076) b(.924)	b	b	b(.593) d(.407)
Idh-2	c	c	c	c (n = 28)	c	c	a(.020) c(.980)	c	c	c	c	c
Ldh (heart)	f	f	f	f (n = 29)	f	a(.100) f(.900)	a(.020) f(.980)	f	f	f	f	f
Ldh (muscle)	h	h	h	h	h(.970) i(.030)	h	e(.020) g(.980)	g(.750) h(.250)	g	g	c(.036) g(.964)	g

	P. variolatus							*P. ocmulgee*				
Population No.	23	24	25	26	27	28	29	30	31	32	33	34
n:	30	33	30	30	33	25	25	26	33	30	28	27
Locus												
Lap	b(.800) c(.200)	b(.469) c(.531) (n=32)	b(.867) c(.133)	b(.500) c(.500) (n=28)	a(.143) b(.786) c(.071) (n=35)	a(.960) c(.040)	a(.600) c(.400) (n=24)	a	a(.924) c(.076)	a	a	a
Mdh-1	b (n=27)	b	b	b	b (n=36)	b	b	b	b	b	b	b
Mdh-2	d	d	d	d	d (n=36)	d (n=24)	d(.980) e(.020)	d	d (n=30)	d	d	d
Pep	c(.833) d(.150) f(.017)	c(.848) d(.152)	c	d	c(.071) d(.929) (n=35)	d(.580) f(.420)	d	d(.981) g(.019)	d	d	d	d
Pgi	g	g	g	g	d(.015) g(.985)	g	g	g	g	g	g	g
Pgm	c	c	c	c	c	c	c	c	c (n=32)	c	c	c
Pt-1	b	b	b	b (n=26)	b	b	b	b	b	b	b	b

Pt-2	a	a	a (n=29)	a	a	a	a	a	a (n=32)	a	a (n=26)	a
6-Pgd	c	c	c	c	c	b(.120) d(.880)	b(.040) c(.960)	b(.635) d(.365)	a(.455) d(.545)	b(.450) d(.550)	b(.161) d(.839)	b(.907) d(.093)
Trf	o (n=44)	f(.470) o(.530)	f(.341) o(.659) (n=41)	o (n=44)	o(.996) q(.004) (n=121)	k(.160) o(.840)	i(.020) j(.020) o(.960)	h(.096) i(.904)	h(.300) i(.700) (n=40)	i (n=109)	i (n=44)	h(.426) i(.574)
H	.050	.075	.069	.036	.066	.102	.093	.078	.082	.059	.038	.060

P. glutinosus

Population No.	35	36	37	38	39	40	41	42	43
n:	23	29	27	32	31	30	16	29	30
Locus									
Alb	c(.935) e(.065)	b(.708) c(.292) (n=24)	c (n=26)	d(.952) f(.048) (n=31)	d	d (n=67)	d (n=25)	d (n=123)	d (n=34)
α-Gpd	b	a(.086) b(.914)	b	b(.984) d(.016) (n=31)	b(.554) e(.446) (n=28)	b	b	b	b
Est	m(.304) p(.370) q(.022) t(.283) x(.022)	h(.017) k(.345) p(.517) t(.070) v(.017) z(.034)	l(.056) p(.593) t(.352)	l(.031) p(.891) t(.047) z(.016) dd(.016)	p(.290) t(.710)	p(.883) t(.117)	p(.406) t(.594)	k(.759) p(.241)	p(.800) t(.200)

	P. glutinosus								
Population No.	35	36	37	38	39	40	41	42	43
n:	23	29	27	32	31	30	16	29	30
Locus									
Fum	b	b	b	b	b (n = 30)	b	b	b	b
Gdh	b	b	b	b (n = 30)	b	b	b	b	b
Got-1	j	h(.017) j(.983)	j	j(.938) k(.063)	j	j	j	j	j
Got-2	e	e	e	e	e	e	e	e	e
Idh-1	b	b	b	b(.469) e(.531)	b	b	b	b	b
Idh-2	?(.022) c(.978)	c	c	c(.422) d(.578)	c	c	c	c	c
Ldh (heart)	f	f	f	d(.125) f(.875)	d(.879) f(.121) (n = 29)	d	d	d	d(.433) f(.567)
Ldh (muscle)	g	g	g	g	g (n = 27)	g	g	g	g

Lap	a	a	a	a	a	a	a	a	a
Mdh-1	b	b	b	b	b (n = 28)	b	b	b	b
Mdh-2	d	d	d	d	d (n = 25)	d	d	d	d
Pep	d	a(.052) d(.948)	b(.037) d(.963)	d	d	d	d	d	d
Pgi	g	g	b(.037) g(.963)	f(.016) g(.984)	g	g	g	g	g
Pgm	c(.652) d(.348)	c	c	c	c	c (n = 27)	c(.906) d(.094)	c(.793) d(.207)	c
Pt-1	b	b(.845) c(.155)	b	b	b	b	b	b	b
Pt-2	a	a	a	a	a	a	a	a	a
6-Pgd	d(.457) e(.543)	b(.574) d(.426) (n = 27)	a(.019) b(.346) d(.635) (n = 26)	b(.100) d(.900) (n = 30)	d	d	d	d	d
Trf	i(.239) l(.739)	f(.261) h(.065)	f(.167) k(.722)	d(.188) f(.021)	h(.274) i(.629)	i(.617) l(.383)	h(.283) o(.717)	h(.356) o(.644)	h(.250) k(.721)

	P. glutinosus									
Population No.	35	36	37	38	39	40	41	42	43	
n:	23	29	27	32	31	30	16	29	30	
Locus										
	o(.022)	i(.130) j(.022) k(.130) l(.152) m(.043) n(.022) o(.152) q(.022) (n=23)	o(.111)	h(.313) i(.021) k(.208) n(.208) q(.042) (n=24)	j(.016) k(.081)	(n=64)	(n=23)	(n=101)	n(.029) (n=34)	
H	.100	.132	.072	.120	.075	.031	.048	.052	.056	

	P. glutinosus									
Population No.	44	45	46	47	48	49	50	51	52	53
n:	29	30	30	29	30	30	30	30	30	25
Locus										
Alb	d (n=110)	d (n=53)	d (n=53)	d	d (n=79)	d (n=54)	d(.947) f(.053) (n=95)	d (n=119)	d (n=98)	d (n=35)
α-Gpd	b(.900) d(.100) (n=30)	b	b	b (n=27)	b (n=27)	b	b	b (n=29)	b	b (n=36)

Est	k(.569) p(.431)	p(.483) t(.517)	k(.034) p(.103) t(.466) y(.224) z(.138) dd(.034) (n = 29)	p(.587) s(.018) t(.395) (n = 28)	p(.389) t(.611) (n = 27)	p(.733) t(.250) y(.017)	p(.800) t(.200)	p(.500) t(.500)	t	p(.080) t(.920)
Fum	b	b(.667) c(.333) (n = 27)	b	b	a(.017) b(.983)	b	b (n = 26)	b	b	b
Gdh	b	b	b	b	b	b	b	b	b	b (n = 36)
Got-1	j (n = 30)	j	h(.133) j(.850) l(.017)	j	d(.100) j(.900)	a(.067) j(.933)	j	a(.083) h(.017) j(.900)	j	j
Got-2	e (n = 27)	e	e	e	e	e	e	e	e	e
Idh-1	b	b	b	b	b	b	b	b (n = 29)	b	b(.700) f(.300)
Idh-2	c	c(.883) e(.117)	c (n = 28)	c	c(.717) e(.283)	c(.983) e(.017)	c	c(.948) e(.052) (n = 29)	c	c

	P. glutinosus									
Population No.	44	45	46	47	48	49	50	51	52	53
n:	29	30	30	29	30	30	30	30	30	25
Locus										
Ldh (heart)	d(.828) f(.172)	d(.533) f(.467)	a(.050) f(.950)	f	f	d(.033) f(.967)	d(.083) f(.917)	f	d(.083) f(.917)	d(.060) f(.940)
Ldh (muscle)	g	g	g	g (n=27)	g	g	g	g	g	g
Lap	a(.981) c(.019) (n=27)	a	a(.966) c(.034) (n=29)	a (n=28)	a (n=28)	a(.983) c(.017)	a	a	a	a
Mdh-1	b (n=27)	b	b	b	b	b	b	b	b	b
Mdh-2	d (n=27)	d	d	d	d	d	d	d	d	d
Pep	d	d	d	d (n=28)	d	d	d	d	d	d(.980) e(.020)
Pgi	g	g	c(.150) g(.850)	g (n=28)	g	g	g	g	g	g
Pgm	c(.586) d(.414)	c(.800) d(.200)	c(.967) d(.033)	c	c	c(.883) d(.117)	c	c	c(.917) d(.083)	c

Pt-1	b (n=31)	b	b	b	b	b	b	b	b	b (n=24)
Pt-2	a	a (n=26)	a	a (n=28)	a	a	a	a	a	a
6-Pgd	d	d	b(.267) d(.733)	d (n=27)	d (n=27)	d	d (n=29)	d (n=28)	d	d (n=24)
Trf	f(.391) i(.050) k(.559) (n=110)	d(.250) f(.025) g(.225) h(.125) i(.025) k(.100) n(.050) o(.150) s(.050) (n=20)	d(.054) g(.036) h(.018) i(.161) k(.482) l(.036) m(.143) n(.018) o(.054) (n=28)	f(.828) o(.172)	f (n=79)	f(.367) g(.233) o(.400)	f(.441) o(.559) (n=93)	f(.012) g(.595) l(.006) o(.387) (n=84)	d(.015) f(.469) i(.439) k(.077) (n=98)	f(.500) o(.500) (n=35)
H	.091	.127	.116	.036	.050	.069	.048	.058	.040	.055

	P. glutinosus									
Population No.	54	55	56	57	58	59	60	61	62	63
n:	11	31	26	30	31	30	27	32	36	28
Locus										
Alb	d (n=16)	d (n=141)	d (n=29)	d (n=48)	d (n=74)	d (n=33)	d	d (n=116)	d (n=101)	d

	P. glutinosus									
Population No.	54	55	56	57	58	59	60	61	62	63
n:	11	31	26	30	31	30	27	32	36	28
Locus										
α-Gpd	b	b (n=30)	b	b	b	b	b	b	b(.986) c(.014) (n=35)	b(.964) e(.036)
Est	p(.227) t(.773)	t	p(.096) t(.904)	p(.033) t(.967)	p(.226) t(.774)	p(.250) t(.750)	p(.093) t(.907)	p(.203) t(.796)	p(.097) t(.903)	p(.125) t(.875)
Fum	b	b	b	b	b	b	b	b	b	b
Gdh	b	b	b	b	b	b	b	b	b	b
Got-1	h(.091) j(.909)	j	j	j	j	j	j	d(.031) j(.969)	d(.014) j(.986)	d(.018) j(.982)
Got-2	e	e	e	e	e	e	e	e	e	d(.018) e(.982)
Idh-1	b	b	b	b	b	b	b	b	b	b(.964) f(.036)
Idh-2	c	c	c(.904) e(.096)	c(.967) e(.033)	c(.968) e(.032)	c(.968) e(.032)	c(.833) e(.167)	c(.891) e(.109)	c(.944) e(.056)	c

Ldh (heart)	f	f	d(.173) f(.827)	d(.117) f(.883)	d(.242) f(.758)	d(.017) f(.983)	d(.204) f(.796)	d(.172) f(.828)	d(.097) f(.903)	d(.036) f(.964)
Ldh (muscle)	g	g (n = 29)	g	g	g	g	g (n = 26)	g	g	g
Lap	a	a	a	a	a	a	a	a	a	a(.982) c(.018)
Mdh-1	b	b	b	b	b	b	b	b (n = 31)	b (n = 19)	b
Mdh-2	d	d	d	d	d	d	d	d	d	d
Pep	d	d	d	d	d	d	d	d	d	d
Pgi	g	g	g	g	g (n = 26)	g	g	g (n = 29)	c(.014) g(.986)	g
Pgm	c	c	c	c	c	c	c	c	c	c
Pt-1	b	b	b	b	b	b	b (n = 17)	b	b (n = 35)	b
Pt-2	a	a	a	a (n = 28)	a	a	a	a (n = 31)	a	a (n = 27)

	P. glutinosus									
Population No.	54	55	56	57	58	59	60	61	62	63
n:	11	31	26	30	31	30	27	32	36	28
Locus										
6-Pgd	d	d	d	d	d	d	d	d	d (n=35)	b(.018) d(.982)
Trf	f(.688) o(.313) (n=16)	f(.798) o(.202) (n=126)	f(.534) o(.466) (n=29)	f(.635) o(.365) (n=48)	d(.007) f(.649) o(.345) (n=74)	f(.182) o(.818) (n=33)	f(.259) o(.741)	f(.177) o(.823) (n=116)	f(.323) o(.677) (n=99)	f(.482) o(.518)
H	.043	.015	.051	.036	.056	.034	.053	.053	.044	.049

	P. glutinosus									
Population No.	64	65	66	67	68	69	70	71	72	73	74
n:	29	30	33	33	29	29	30	30	31	34	31
Locus											
Alb	d (n=41)	d (n=40)	d (n=37)	d (n=34)	d (n=53)	d (n=116)	d (n=107)	d (n=71)	d(.570) h(.430) (n=93)	d(.238) e(.180) h(.582) (n=61)	e (n=30)
α-Gpd	b (n=28)	b(.967) e(.033)	b	b (n=32)	b (n=26)	b(.983) d(.017)	b	b	b (n=30)	b	b

Est	p(.259) t(.741)	p(.233) t(.767)	p(.076) t(.924)	t	p(.172) t(.828)	p(.052) t(.948)	p(.300) t(.700)	p(.217) t(.783)	e(.065) k(.161) p(.210) t(.387) y(.145) dd(.032) (n=27)	e(.015) k(.074) m(.029) p(.706) t(.088) y(.088)	p(.500) t(.500)
Fum	b	b	b	b	b	b	b	b(.983) c(.017)	b	b (n=30)	b
Gdh	b	b	b	b(.985) d(.015)	b	b	b	b	a(.375) b(.625) (n=28)	b	b
Got-1	h(.052) j(.948)	d(.017) j(.983)	d(.136) j(.864)	h(.016) j(.984) (n=32)	j	j	d(.050) j(.950)	j	c(.070) h(.372) j(.558) (n=43)	h(.382) j(.485) l(.132)	h(.048) j(.952)
Got-2	e	d(.033) e(.967)	e	e (n=32)	e	e	e	e	e (n=43)	e	e
Idh-1	b	b(.983) f(.017)	b	b	b	b	b	b	b	b	b(.790) e(.210)
Idh-2	c	c	c	c	c	c	c(.946) e(.054) (n=28)	c	c	c(.985) e(.015)	c

	P. glutinosus										
Population No.	64	65	66	67	68	69	70	71	72	73	74
n:	29	30	33	33	29	29	30	30	31	34	31
Locus											
Ldh (heart)	d(.052) f(.948)	f	d(.030) f(.970)	f	d(.655) f(.345)	d(.500) f(.500)	d(.650) f(.350)	f	f	f	d(.983) f(.017)
Ldh (muscle)	g	f(.017) g(.983)	g	g	g	g	g	g(.883) h(.117)	g(.968) i(.032)	a(.015) g(.985)	g
Lap	a	a	a(.909) c(.091)	a	a	a	a	a	a(.538) c(.462) (n=26)	a(.941) c(.059)	a
Mdh-1	b	b (n=28)	b	b	b	b	b	b	b	b (n=30)	b
Mdh-2	d	d	d	d	d	d	d (n=21)	d	d	d (n=33)	d
Pep	d (n=28)	d	d	d	d	d	d	d	d	b(.074) d(.926)	d
Pgi	g	a(.017) g(.983)	g	g	g	g	g	g	g	g (n=30)	c(.387) e(.032) g(.581)
Pgm	c (n=23)	c(.983) d(.017)	c	c (n=30)	c	c	c	c	c	c (n=29)	c(.758) d(.242)

Pt-1	b	b	b	b (n=32)	b	b	b	b	b	b	b	
Pt-2	a	a	a	a (n=32)	a	a	a	a	a	a (n=30)	a	
6-Pgd	d	d	d	d (n=32)	d	d	d	b(.879) d(.121) (n=29)	b(.354) d(.646) (n=24)	b(.147) d(.853)	d (n=26)	
Trf	f(.415) o(.585) (n=41)	f(.450) o(.550) (n=40)	f(.405) o(.595) (n=37)	f(.059) o(.941) (n=34)	g(.029) k(.202) o(.769) (n=52)	f(.407) o(.593) (n=113)	f(.841) o(.159) (n=107)	f(.371) i(.078) l(.543) ?(.009)	h(.269) k(.154) l(.067) m(.019) o(.490) (n=52)	d(.067) h(.744) i(.033) l(.156) (n=45)	d(.017) g(.583) k(.200) o(.200) (n=30)	
H	.049	.052	.049	.008	.050	.051	.061	.062	.179	.119	.110	

	P. kiamichi		*P. mississippi*										
Population No.	75	76	77	78	79	80	81	82	83	84	85	86	87
n:	34	39	36	27	31	30	28	30	30	30	22	28	26
Locus													
Alb	b(.014) e(.986) (n=72)	b(.012) e(.988) (n=164)	d	d (n=109)	d (n=47)	d(.929) g(.058) j(.013) (n=112)	d (n=101)	d(.971) f(.029) (n=68)	d(.961) f(.039) (n=76)	d (n=44)	d(.842) e(.044) f(.114) (n=57)	d (n=38)	d (n=89)

	P. kiamichi		*P. mississippi*										
Population No.	75	76	77	78	79	80	81	82	83	84	85	86	87
n:	34	39	36	27	31	30	28	30	30	30	22	28	26
Locus													
α-Gpd	b	b	b	b (n=22)	b	b	b	b	b	b (n=25)	b	b (n=27)	?(.019) b(.981)
Est	h(.029) p(.309) t(.662)	p(.090) t(.910)	g(.029) i(.014) k(.257) m(.229) p(.357) t(.029) u(.029) y(.057) (n=35)	h(.019) k(.019) m(.167) p(.537) t(.259)	k(.065) p(.919) t(.016)	t	t	m(.017) o(.017) t(.967)	t	p(.150) t(.850)	t	t	p(.130) t(.556) dd(.241) hh(.074) (n=27)
Fum	b(.606) c(.394) (n=33)	b	b	b	b	b	b	b	b	b	b (n=31)	b	b(.981) c(.019)
Gdh	b	b	b	b	b	b	b	b	b	b	b	b	b
Got-1	b(.618) g(.263) j(.118) (n=76)	b(.500) g(.316) j(.184)	h(.222) j(.778)	j	j	j	j (n=42)	j (n=24)	j	j(.983) k(.017)	j	j	j(.615) k(.385)

Got-2	e	e (n=23)	?(.014) e(.986)	e	e	e	e (n=43)	e (n=29)	e	e	e	e	e
Idh-1	b	b	b(.958) g(.042)	b	b	b	b	b	b	b	b	b	b
Idh-2	b(.015) c(.985)	c(.987) d(.013)	c	c(.963) d(.037)	c	c	c	c	c	c	c	c	c
Ldh (heart)	f	f (n=37)	f	f (n=26)	f	f	f	f(.967) g(.033)	f	f	f(.891) i(.109)	f(.982) i(.108)	f
Ldh (muscle)	h	h (n=37)	h	f(.093) h(.907)	h (n=30)	g	g	h	h	h	h	h	h
Lap	a(.516) c(.484) (n=31)	a(.125) c(.875) (n=36)	a (n=29)	a (n=26)	a	a	a	a	a	a	a	a	a
Mdh-1	b	b	b	b (n=26)	b	b	b	b (n=23)	b	b	b	b	b
Mdh-2	d (n=30)	d (n=23)	d	b(.148) d(.833) f(.019)	d	d	d	d (n=29)	d	d	d	d	d (n=25)

	P. kiamichi		*P. mississippi*										
Population No.	75	76	77	78	79	80	81	82	83	84	85	86	87
n:	34	39	36	27	31	30	28	30	30	30	22	28	26
Locus													
Pep	d	d (n=34)	d(.972) f(.028)	a(.037) d(.963)	b(.016) d(.984)	d	d (n=27)	d(.983) f(.017)	d	d	d	d	b(.538) d(.462)
Pgi	g	g (n=23)	c(.097) g(.903)	g (n=25)	d(.306) g(.694)	g	g	c(.034) g(.966)	g	g	g	g	g
Pgm	c	c (n=23)	c	c(.981) d(.019)	c (n=21)	c	a(.036) c(.964)	c (n=29)	c	c(.900) d(.100)	c	c	c (n=23)
Pt-1	c	c	b(.111) c(.889)	c (n=26)	c	b(.017) c(.983)	c	c	c	c	b(.031) c(.969)	c	c
Pt-2	a	a	a (n=34)	a (n=22)	a	a	a (n=27)	a	a	a	a (n=30)	a	a (n=25)
6-Pgd	b(.469) d(.531) (n=32)	b(.018) d(.931) e(.052) (n=28)	b(.227) d(.727) e(.045) (n=22)	b(.519) d(.481) (n=26)	b(.097) d(.903)	b(.933) d(.067)	a(.036) b(.964)	a(.042) b(.958) (n=24)	b	b(.767) d(.233)	b	b	b
Trf	h(.202)	h(.168)	d(.056)	i(.814)	g(.012)	h(.775)	h(.589)	h(.478)	h(.545)	h(.271)	h(.509)	h(.020)	l

		i(.393) j(.333) m(.071) (n=42)	i(.450) j(.045) m(.282) o(.005) q(.050) (n=101)	f(.111) g(.019) h(.389) j(.130) k(.019) l(.241) o(.037) (n=27)	l(.186) (n=78)	i(.547) k(.047) m(.105) o(.291) (n=43)	l(.054) o(.005) q(.167) (n=102)	l(.371) q(.040) (n=101)	l(.154) o(.221) q(.147) (n=68)	l(.422) o(.032) (n=77)	i(.214) k(.300) l(.029) o(.057) q(.129) (n=35)	l(.491) (n=57)	l(.980) (n=49)	(n=50)
H		.146	.083	.127	.093	.063	.030	.030	.047	.027	.073	.047	.003	.075

	P. kisatchie			*P. sequoyah*	*P. albagula*	*P. grobmani*								
Population No.	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
n:	10	28	21	29	36	17	28	12	38	31	28	30	25	25	30
Locus															
Alb	d	c (n=62)	c (n=24)	d (n=106)	a(.010) c(.620) d(.030) e(.340) (n=50)	e(.529) g(.471)	e(.969) ?(.031) (n=16)	e	e	e(.984) h(.016) (n=62)	e (n=46)	e (n=43)	e(.820) g(.180)	e(.960) i(.040)	e (n=72)
α-Gpd	b	b(.982) d(.018)	b	b	b	b	b	b	b (n=36)	b (n=27)	b	b	b	b	b
Est	1	1	1	k(.276) p(.724)	p(.722) t(.278)	t	p(.250) t(.750)	p(.125) t(.875)	t	k(.032) p(.758) t(.210)	p(.875) t(.107) dd(.018)	p	k(.440) p(.300) t(.240) y(.020)	k(.020) p(.220) t(.700) z(.060)	f(.117) l(.467) p(.150) t(.267)

	P. kisatchie			*P. sequoyah*	*P. albagula*	*P. grobmani*								
Population No.	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
n:	10	28	21	29	36	17	28	12	38	31	28	30	25	25	30
Locus															
Fum	b	b (n=24)	b	b	b	b	b	b	b	b	b	b	b(.640) c(.360)	b	b(.117) c(.883)
Gdh	b	b	b	b	b	b	b	b	b (n=37)	b (n=30)	b	b	b	b	b
Got-1	h	h	h	h(.983) j(.017)	e(.111) g(.806) j(.083)	j(.176) k(.824)	j(.946) k(.054)	j	j(.263) k(.737)	j	j	j	j(.980) k(.020)	j	j(.517) k(.483)
Got-2	e	e	e	e	e (n=35)	e	e	e	e	e	e	e	e	e	e
Idh-1	b	b (n=27)	b	b	b	b	b	b	b	b	b	b	b	b	b
Idh-2	c	c (n=27)	c	c(.931) e(.069)	c	b	c(.964) d(.036)	c	b	a(.048) c(,952)	c	c	c	c	a(.017) c(.983)
Ldh (heart)	f	f (n=27)	f	f	f	f	f	f	f	f (n=30)	f	f	f	f	f
Ldh (muscle)	h	h	h	h	h	h	h	h	h	g (n=30)	g(.357) h(.643)	g(.350) h(.650)	h	h	h

Lap	a	a	a	a	a(.766) c(.234) (n = 32)	a	a	a	a	a (n = 29)	a	a	a	a	a
Mdh-1	a(.050) b(.950)	b	b	b (n = 28)	b (n = 17)	b	b	b (n = 11)	b	b	b	b	b	b	b
Mdh-2	d	d	d	a(.793) d(.207)	d	d (n = 16)	d	d	d (n = 36)	d(.984) f(.016)	d	d	d	d	d(.967) e(.033)
Pep	d	d (n = 25)	d	d	d(.958) f(.042)	d	d	d	d	d	d(.661) f(.339)	d	d(.920 f(.080)	d	d
Pgi	g	g	g	g (n = 25)	g	g	g	g	g	c(.032) g(.968)	g	g	c(.040) g(.960)	g	g
Pgm	c	b(.018) c(.982)	c	c	c(.986) d(.014)	c	c	c	c	c	c	c	c	c	c
Pt-1	c	c	c	c	b	b	b	b	b	b	b	b	b	b	b
Pt-2	a	a (n = 27)	a	a (n = 34)	a (n = 34)	a	a (n = 22)	a	a	a	a	a	a	a	a
6-Pgd	b	b	b	d	b(.424) d(.576) (n = 33)	b	b	b (n = 11)	b	b(.500) d(.500)	b(.964) d(.036)	b	a(.020) b(.900) d(.080)	b	b(.433) d(.567)

	P. kisatchie			*P. sequoyah*	*P. albagula*	*P. grobmani*								
Population No.	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
n:	10	28	21	29	36	17	28	12	38	31	28	30	25	25	30
Locus															
Trf	k	k (n=62)	k (n=24)	k (n=106)	f(.056) h(.083) j(.306) k(.458) l(.097)	l	j(.052) l(.948) (n=29)	l	i(.026) l(.974)	i(.927) l(.073) (n=62)	j(.333) l(.667) (n=45)	i(.595) k(.270) l(.068) m(.054) o(.014) (n=37)	d(.060) f(.040) g(.020) i(.540) k(.280) n(.060)	i(.396) j(.563) l(.042) (n=24)	g(.069) i(.446) j(.392) k(.077) l(.008) m(.008) (n=65)
H	.004	.003	0	.040	.130	.036	.032	.010	.020	.056	.075	.046	.113	.048	.118

	P. cylindraceus												
Population No.	103	104	105	106	107	108	109	110	111	112	113	114	115
n:	29	26	27	30	32	30	32	30	30	26	27	31	31
Locus													
Alb	e (n=54)	e (n=112)	e (n=27)	e (n=53)	e (n=56)	e (n=87)	e (n=100)	e (n=91)	e(.807) h(.193) (n=133)	d (n=35)	d (n=71)	d (n=116)	c(.984) d(.016)
α-Gpd	b	b	b	b	b (n=29)	b (n=29)	b	b	b	b	b	b	b

Est	e(.603) j(.241) p(.052) t(.103)	e(.442) j(.154) p(.365) t(.038)	e(.241) p(.759)	e(.283) p(.717)	e(.359) p(.641)	e(.150) p(.850)	e(.281) p(.719)	e(.133) j(.150) p(.717)	e(.717) j(.283)	e(.808) p(.192)	e(.963) j(.037)	e	e(.952) j(.048)
Fum	b	b	b	b	b	b	b	b	b	b	b	b	b
Gdh	b	b	b	b	b (n=28)	b	b	b	b	b	b	b	b
Got-1	h(.862) j(.138)	h(.885) j(.115)	h	h	h (n=31)	h	h	h	h	h	h	h	h
Got-2	e	e	e	e	e	e	e	e	e	a(.212) e(.788)	e	e	e
Idh-1	b	b(.962) g(.038)	b	b	b (n=29)	b	b	b	b(.950) e(.050)	b	b	b	b
Idh-2	c (n=24)	c(.942) e(.058)	c	c	c (n=29)	c	c	c	c	c	c(.944) a(.056)	c	c
Ldh (heart)	f	f	f	f	f	f	e(.047) f(.953)	f	f	f	f	f	f
Ldh (muscle)	h	h	h	h	h	h	h	h	h	h	h	h	h

	P. cylindraceus												
Population No.	103	104	105	106	107	108	109	110	111	112	113	114	115
n:	29	26	27	30	32	30	32	30	30	26	27	31	31
Locus													
Lap	a(.967) c(.033)	a (n=25)	a	a	a	a	a	a (n=20)	a	a	a	a	a
Mdh-1	b	a(.560) b(.440) (n=25)	a	a(.833) b(.167)	a(.719) b(.281)	a(.800) b(.200)	a(.548) b(.452) (n=31)	a(.483) b(.517)	a(.183) b(.817)	b	b	b	b
Mdh-2	d	d	d	c(.017) d(.983)	d	d	d	d	d	d	d (n=26)	d(.984) e(.016)	d
Pep	d	d	d	d	d	d	d	d	d	d	d (n=26)	d	b(.065) d(.919) e(.016)
Pgi	g	g	g	g	g	g	g (n=25)	g	g	g	g	g	g
Pgm	c (n=27)	c	c	c	c	c	c	c	c	c	c	c	a(.081) c(.919)
Pt-1	b	b	b	b	b	b	b	b	b	b	b	b	b

Pt-2	a (n=21)	a (n=20)	a	a	a	a	a (n=29)	a	a (n=21)	a (n=25)	a	a (n=29)	a
6-Pgd	b(.983) d(.017)	b(.981) d(.019)	b	b	b (n=25)	b	b	b	b	b(.462) d(.519) e(.019)	b(.704) d(.296)	b(.613) d(.387)	b
Trf	h(.492) o(.508) (n=62)	f(.186) g(.379) i(.243) j(.029) l(.014) o(.150) (n=70)	i	i (n=53)	i (n=56)	i (n=88)	i (n=97)	i (n=91)	f(.019) h(.038) i(.942) (n=52)	b(.115) h(.077) i(.808)	b(.063) j(.937) (n=71)	j (n=115)	a(.048) b(.596) d(.032) g(.065) i(.258)
H	.064	.105	.017	.033	.039	.026	.045	.043	.056	.068	.032	.023	.045

P. albagula

Population No.	116	117	118	119	120	121	122	123	124	125	126	127
n:	27	30	28	30	30	30	25	26	31	30	30	14
Locus												
Alb	e(.612) i(.388) (n=236)	e (n=87)	e (n=33)	e (n=84)	e (n=80)	e(.988) i(.012) (n=82)	e	e(.906) g(.094)	e(.956) g(.044) (n=91)	e (n=87)	e (n=92)	e

	P. albagula											
Population No.	116	117	118	119	120	121	122	123	124	125	126	127
n:	27	30	28	30	30	30	25	26	31	30	30	14
Locus												
α-Gpd	b	b	b	b	b	b	b (n=24)	b	b	b	b	b
Est	k(.468) o(.387) t(.016) u(.016) z(.113) (n=31)	t(.517) z(.133) cc(.017) ff(.333)	r(.036) t(.036) cc(.929) (n=28)	l(.617) cc(.383)	p(.267) t(.300) z(.017) cc(.417)	p(.167) t(.833)	t(.060) z(.940)	v(.423) z(.577)	t (n=32)	t	p(.483) t(.467) z(.050)	p(.964) t(.036)
Fum	b (n=23)	b	b	b	b	b	b	b (n=16)	b (n=28)	b	b	b
Gdh	b	b	b	b	b	b	b	b	b	b	b	b
Got-1	h(.593) j(.407)	h(.117) j(.883)	j	j	j	j	j	j	j	j	j	j
Got-2	e	d(.300) e(.700)	e	e	b(.017) e(.983)	e	e	e	e	e	c(.167) e(.833)	e
Idh-1	b	b(.900) e(.100)	b	b	b	b	b	b	b	b	b(.850) e(.150)	b

Idh-2	c	c	c	c	c	a(.067) c(.933)	c	c	a(.016) c(.984)	a(.183) c(.817)	c	c
Ldh (heart)	f	f	f	f	f	f	f	f	f	f	f	f
Ldh (muscle)	h	b(.017) h(.983)	h	h	h	h	h	h	h	e(.052) h(.948) (n = 29)	h	h
Lap	a	a	a	a (n = 28)	a	a	a	a	a	a	a	a
Mdh-1	b (n = 28)	b	b	b	b	b	b	b(.673) c(.327)	b	b	b	a(.036) b(.964)
Mdh-2	b(.729) c(.063) d(.208) (n = 28)	d	d	d	d	d	d	d	d	d	d	d
Pep	b (n = 30)	b(.417) d(.583)	b	b	b	b	b	b	b(.032) d(.968)	d	d	d
Pgi	g (n = 24)	g	g(.982) h(.018)	g	g	g(.981) h(.019) (n = 26)	g	c(.031) g(.969) (n = 16)	g	g	g	g
Pgm	c	c	c	c	c	c(.967) g(.033)	c	c	c	c	c	c

	P. albagula											
Population No.	116	117	118	119	120	121	122	123	124	125	126	127
n:	27	30	28	30	30	30	25	26	31	30	30	14
Locus												
Pt-1	b	b	b	b	b	a(.017) b(.983)	b	b	b	b	b	b
Pt-2	f(.608) h(.392) (n=26)	h (n=29)	f(.319) h(.681)	f(.106) h(.894)	f(.069) h(.931)	h	f(.510) h(.490)	f(.250) h(.750) (n=16)	f(.423) h(.577) (n=20)	f	a (n=22)	a
6-Pgd	d (n=29)	d	d	d	d	d	d	d	d (n=30)	d	d (n=29)	d
Trf	k(.479) o(.521) (n=236)	k (n=87)	k (n=33)	i(.024) j(.976) (n=84)	k (n=79)	i(.098) k(.902) (n=82)	k(.420) o(.580)	k	k(.911) n(.089) (n=90)	j(.017) k(.913) n(.070) (n=86)	k (n=76)	k
H	.135	.088	.028	.032	.038	.034	.050	.070	.038	.025	.049	.006

	P. savannah	*P. aureolus*			*P. kentucki*			
Population No.	128	129	130	131	132	133	134	135
n:	30	30	33	33	32	30	32	33
Locus								
Alb	h (n=31)	d(.100) e(.900)	e (n=92)	d (n=37)	a(.013) b(.988) (n=80)	b (n=65)	a	a (n=61)
α-Gpd	b	b (n=29)	b	b	b(.859) e(.141)	b(.817) e(.183)	b(.563) e(.438)	b(.970) e(.030)
Est	p	q(.033) s(.617) t(.100) w(.250)	o(.042) p(.069) t(.888) (n=36)	e(.015) l(.106) n(.030) o(.015) p(.348) r(.197) t(.288)	p(.016) t(.250) x(.172) dd(.563)	o(.250) t(.567) u(.017) v(.017) x(.150)	p(.672) t(.328)	q(.015) s(.030) t(.939) x(.015)
Fum	b	a(.033) b(.967)	a(.343) b(.657) (n=35)	b (n=32)	b (n=30)	b	b	b
Gdh	b	b	b	b (n=32)	b	b	b	b
Got-1	j	j	h(.030) j(.970)	j	a(.438) d(.563)	a(.183) d(.783) j(.033)	a(.281) d(.719)	a(.485) d(.515)

	P. savannah	*P. aureolus*			*P. kentucki*			
Population No.	128	129	130	131	132	133	134	135
n:	30	30	33	33	32	30	32	33
Locus								
Got-2	e	e	e	c(.015) e(.985)	d(.984) e(.016)	d	d(.344) e(.656)	d(.924) e(.076)
Idh-1	d	b	b(.485) e(.515)	b(.742) e(.152) g(.106)	b(.406) f(.594)	b(.983) f(.017)	b(.094) f(.891) h(.016)	b(.742) f(.258)
Idh-2	c	c(.383) d(.617)	a(.028) c(.292) d(.681) (n = 35)	c(.045) d(.955)	c(.031) e(.969)	e	c(.063) e(.938)	c(.061) e(.939)
Ldh (heart)	f	f	f (n = 36)	d(.015) f(.985)	d(.031) f(.969)	f	f	f
Ldh (muscle)	h	f(.897) h(.103) (n = 29)	f(.861) h(.139) (n = 36)	f(.985) h(.015)	h	h	g(.031) h(.969)	h
Lap	a	a (n = 29)	a (n = 36)	a	a	a	a	a

Mdh-1	b	b	b	b	b	b	b (n=31)	b (n=30)
Mdh-2	d	b(.017) d(.983)	d	d	d	d	d (n=31)	d
Pep	d	d	d	d	b(.297) d(.672) e(.031)	b(.550) d(.450)	b(.547) d(.453)	b(.530) d(.470)
Pgi	g	g (n=29)	e(.014) g(.986) (n=36)	g	g	f(.033) g(.967)	g	g
Pgm	c	c(.828) d(.172) (n=29)	c (n=36)	c	c	b(.017) c(.933) e(.050)	c	c
Pt-1	b	b(.603) c(.397) (n=29)	c	c	b	b	b	b
Pt-2	a	b(.121) d(.879) (n=29)	d	b(.017) d(.983) (n=30)	g	a(.017) g(.983)	g	g (n=32)
6-Pgd	c	b(.946) d(.054) (n=28)	a(.015) b(.758) d(.227)	b(.167) d(.833)	b(.625) c(.375)	b(.237) c(.733)	b(.516) c(.484)	b(.091) c(.909) (n=32)

	P. savannah	*P. aureolus*			*P. kentucki*			
Population No.	128	129	130	131	132	133	134	135
n:	30	30	33	33	32	30	32	33
Locus								
Trf	h (n=31)	g(.346) i(.154) l(.404) m(.077) q(.019) (n=26)	a(.007) b(.033) d(.046) f(.046) g(.099) h(.092) i(.099) j(.191) k(.092) l(.197) m(.039) o(.026) p(.013) r(.013) s(.007) (n=76)	b(.027) f(.973) (n=37)	m(.950) r(.050) (n=80)	m(.992) p(.008) (n=62)	j(.094) m(.906)	m(.475) o(.508) r(.017) (n=59)
H	0	.148	.145	.077	.137	.109	.151	.113

Appendix 3. Immunological distances of the *Plethodon glutinosus* complex

Population			Allele	Antibody					
				New York (pop. 70)		Arkansas (pop. 126)		South Carolina (pop. 21)	
No.	State	County or parish		D	ID	D	ID	D	ID
P. teyahalee									
1	TN	Monroe	c	0.41	2	0.37	5	0.49	10
2	TN	Monroe	c	0.40	4	0.30	4	0.42	4
3	NC	Macon	c	0.45	3	0.35	1	0.47	6
4	NC	Haywood	c	0.43	4	0.34	7	0.47	5
5	NC	Graham	c	0.42	1	0.32	3	0.44	7
6	NC	Madison	c	0.39	3	0.31	3	0.43	10
P. chattahoochee									
7	GA	Union	d	0.22	3	0.27	3	0.29	4
8	GA	Fannin	d	0.22	4	0.30	3	0.31	4
9	GA	Towns	d	0.31	5	0.30	4	0.20	5
10	GA	Rabun	d	0.34	6	0.24	4	0.19	2
			e		6		4		2
P. chlorobryonis									
11	SC	Chesterfield	d	0.31	4	0.25	5	0.17	1
			g		9		10		12
12	SC	Florence	d	0.29	4	0.27	1	0.14	2
13	NC	Columbus	d	0.27	3	0.27	3	0.15	0
14	NC	Craven	d	0.28	2	0.29	1	0.15	7
15	NC	Halifax	d	0.28	4	0.28	4	0.16	1
16	VA	Southampton	d	0.28	1	0.28	4	0.16	1
17	VA	Dinwiddie-Sussex	d	0.28	3	0.28	4	0.15	3
18	NC	Tyrrell	d	0.28	3	0.28	1	0.15	1
19	NC	Pender	d	0.27	1	0.27	3	0.14	3
20	GA	Habersham	d	0.31	9	0.29	9	0.09	5
21	SC	McCormick	b	0.38	3	0.31	2	–	–
			d		0		0		0
22	SC	Aiken	d	0.34	1	0.30	3	0.06	0

Population			Allele	Antibody					
				New York (pop. 70)		Arkansas (pop. 126)		South Carolina (pop. 21)	
No.	State	County or parish		D	ID	D	ID	D	ID
P. variolatus									
23	SC	Charleston-Dorchester	d	0.35	2	0.30	3	0.19	4
24	SC	Jasper	d	0.35	2	0.33	5	0.19	1
25	SC	Allendale	d	0.36	2	0.33	2	0.20	0
26	SC	Jasper	d	0.34	4	0.30	2	0.24	3
27	SC	Berkeley	d	0.26	4	0.24	2	0.23	4
28	GA	Chatham	d	0.18	7	0.14	3	0.20	6
29	GA	Effingham	d	0.16	1	0.24	2	0.28	3
P. ocmulgee									
30	GA	Bacon	d	0.20	3	0.27	0	0.36	7
31	GA	Bulloch	h	0.17	7	0.20	5	0.42	11
32	GA	Wheeler	h	0.19	6	0.23	5	0.38	9
33	GA	Upson	h	0.20	7	0.23	5	0.42	7
34	GA	Long	h	0.26	6	0.30	3	0.39	6
P. glutinosus									
35	TN	Moore	c	0.14	5	0.17	4	0.39	6
36	AL	Etowah	b	0.11	5	0.15	4	0.29	4
			c		4		3		3
37	TN	Bledsoe	c	0.11	3	0.12	5	0.36	9
38	TN	Polk	d	0.09	0	0.16	0	0.36	3
39	KY	Muhlenberg	d	0.04	1	0.20	2	0.42	1
40	IN	Crawford	d	0.05	0	0.19	2	0.42	6
41	IL	Union	d	0.03	0	0.21	3	0.37	3
42	IL	Pope	d	0.04	0	0.21	3	0.38	5
43	KY	Warren	d	0.05	0	0.12	2	0.37	0
44	IN	Parke	d	0.03	0	0.16	1	0.41	6
45	TN	Blount	d	0.03	0	0.16	1	0.37	5
46	AL	Cleburne	d	0.06	1	0.13	0	0.32	5
47	KY	McCreary	d	0.03	0	0.15	0	0.34	4
48	NJ	Union	d	0.03	0	0.16	3	0.37	6
49	VA	Giles	d	0.04	0	0.15	2	0.32	2
50	OH	Meigs	d	0.04	0	0.15	3	0.31	5
51	VA	Russell	d	0.05	0	0.15	1	0.32	7
52	IN	Jackson	d	0.03	0	0.15	3	0.36	8
53	KY	Harlan	d	0.03	0	0.16	0	0.31	2
54	OH	Brown	d	0.02	0	0.15	2	0.32	3
55	MD	Frederick	d	0.02	0	0.16	1	0.35	7

Population			Allele	Antibody					
		County or		New York (pop. 70)		Arkansas (pop. 126)		South Carolina (pop. 21)	
No.	State	parish		D	ID	D	ID	D	ID
56	PA	Cambria	d	0.02	0	0.16	1	0.33	3
57	WV	Preston	d	0.02	1	0.16	2	0.33	5
58	PA	Susquehanna	d	0.01	0	0.15	2	0.33	3
59	NY	Tompkins	d	0.04	2	0.15	1	0.30	4
60	PA	Beaver	d	0.03	2	0.16	4	0.32	2
61	PA	Snyder	d	0.03	0	0.16	3	0.31	4
62	PA	Cameron	d	0.03	0	0.16	1	0.31	4
63	KY	Pike	d	0.03	1	0.15	1	0.31	3
64	WV	Clay	d	0.03	0	0.15	1	0.31	6
65	VA	Wise	d	0.03	0	0.15	1	0.31	3
66	KY	Harlan	d	0.03	0	0.16	2	0.30	3
67	OH	Wayne	d	0.05	0	0.16	3	0.30	4
68	KY	Scott	d	0.03	0	0.18	0	0.33	6
69	PA	Bedford	d	0.01	0	0.17	2	0.34	7
70	NY	Ulster	d	–	–	0.18	1	0.38	4
71	TN	Montgomery	d	0.07	0	0.18	1	0.35	5
72	GA	Henry	d	0.09	3	0.16	3	0.25	1
			h		7		5		7
73	AL	Macon	d	0.11	4	0.14	1	0.33	10
			h		7		5		10
74	TN	Cocke	e	0.10	1	0.15	1	0.44	4
P. kiamichi									
75	OK	Le Flore	e	0.30	4	0.16	0	0.36	6
76	OK	Le Flore	e	0.30	2	0.17	2	0.33	8
P. mississippi									
77	AL	Tuscaloosa	d	0.16	0	0.14	3	0.29	11
78	AL	Lawrence	d	0.19	5	0.17	1	0.35	10
79	MS	Tishomingo	d	0.19	1	0.16	3	0.33	8
80	TN	Henderson	d	0.16	1	0.26	1	0.45	5
81	TN	Shelby	d	0.16	3	0.27	2	0.45	9
82	MS	Winston	d	0.21	3	0.21	2	0.36	8
83	MS	Scott	d	0.22	3	0.21	3	0.38	10
84	MS	Lowndes	d	0.19	4	0.16	3	0.35	10
85	LA	Washington	d	0.22	4	0.20	3	0.38	11
86	MS	Forrest	d	0.23	4	0.22	2	0.39	8
87	AL	Clarke	d	0.27	2	0.25	2	0.37	4
P. kisatchie									
88	AR	Union	d	0.33	5	0.25	3	0.40	7

Population			Allele	Antibody					
				New York (pop. 70)		Arkansas (pop. 126)		South Carolina (pop. 21)	
No.	State	County or parish		D	ID	D	ID	D	ID
89	LA	Winn	c	0.40	4	0.25	1	0.43	3
90	LA	Grant	c	0.40	1	0.25	3	0.43	7
P. sequoyah									
91	OK	McCurtain	d	0.29	2	0.20	3	0.40	14
P. albagula									
92	AR	Montgomery	c	0.21	5	0.09	0	0.29	6
			e		3		0		6
P. grobmani									
93	FL	Hillsborough	e	0.25	3	0.15	3	0.36	4
			g		7		6		8
94	FL	Columbia	e	0.22	3	0.10	2	0.34	8
95	GA	Lanier	e	0.23	5	0.11	0	0.34	8
96	FL	Lake	e	0.26	4	0.09	2	0.37	7
97	AL	Butler	e	0.14	4	0.12	1	0.38	12
98	FL	Leon	e	0.21	4	0.11	1	0.33	5
99	GA	Schley	e	0.21	3	0.09	0	0.35	6
100	AL	Barbour	e	0.22	3	0.08	0	0.29	9
101	GA	Terrell	e	0.21	2	0.09	1	0.32	5
102	FL	Jackson	e	0.25	4	0.11	2	0.32	9
P. cylindraceus									
103	NC	Montgomery	e	0.28	0	0.15	3	0.29	11
104	VA	Grayson-Wythe	e	0.29	4	0.16	3	0.33	9
105	VA	Fairfax	e	0.38	2	0.22	0	0.42	4
106	VA	Bedford	e	0.36	0	0.21	2	0.40	8
107	VA	Botetourt	e	0.35	3	0.20	4	0.39	8
108	VA	Augusta	e	0.36	0	0.20	3	0.40	6
109	WV	Hampshire	e	0.33	3	0.18	4	0.38	9
110	VA	Pittsylvania	e	0.33	3	0.18	2	0.37	5
111	NC	Burke	e	0.32	6	0.19	6	0.34	13
			h		7		6		11
112	SC	Chester	d	0.21	1	0.19	3	0.30	10
113	NC-TN	Madison-Unicoi	d	0.24	3	0.22	3	0.31	7
114	NC-TN	Mitchell-Unicoi	d	0.23	3	0.22	2	0.31	7
115	NC	Henderson	c	0.32	4	0.24	5	0.34	10

Population		Allele	Antibody					
	County or		New York (pop. 70)		Arkansas (pop. 126)		South Carolina (pop. 21)	
No. State	parish		D	ID	D	ID	D	ID
P. albagula								
116 TX	Hays	e	0.35	3	0.20	1	0.31	5
		i		4		5		13
117 AR	Pope	e	0.26	3	0.07	2	0.37	9
118 MO	Iron	e	0.32	4	0.12	0	0.38	11
119 MO	Wayne	e	0.32	4	0.12	0	0.38	14
120 MO	Miller	e	0.30	4	0.11	0	0.37	11
121 AR	Stone	e	0.29	2	0.11	0	0.38	4
122 TX	Travis	e	0.30	0	0.14	0	0.33	7
123 TX	Kerr	e	0.32	5	0.13	0	0.38	8
124 OK	Sequoyah	e	0.21	1	0.05	0	0.37	6
125 OK	Adair	e	0.23	3	0.07	0	0.39	11
126 AR	Garland	e	0.18	1	–	–	0.31	6
127 AR	Dallas	e	0.19	1	0.01	0	0.32	7
P. savannah								
128 GA	Richmond	h	0.32	7	0.21	4	0.36	5
P. aureolus								
129 TN	Monroe	e	0.32	2	0.24	0	0.50	2
130 TN	Monroe	e	0.37	2	0.27	2	0.57	1
131 TN	Polk	d	0.30	3	0.34	2	0.56	0
P. kentucki								
132 VA	Wise	b	0.53	13	0.43	13	0.51	12
133 KY	Harlan	b	0.51	13	0.42	13	0.54	13
134 KY	Pike	a	0.52	7	0.41	7	0.53	8
135 KY	Harlan	a	0.47	8	0.39	9	0.53	8

Index

Albumin (Alb), blood serum, 4, 6, 7, 39, 81
  Polymorphism, 82
Allospecies, 52
Alpha-Glycerophosphate dehydrogenase (α-Gpd), 6, 39
Amino acid sequence difference, 81, 82
Antibody, albumin, 82–92

Batrachoseps, 50
Bolitoglossa occidentalis, 48
Bolitoglossa rufescens, 48
Buffers, 6
  Lithium hydroxide, 6
  Poulik, 6
  Tris-citrate, pH 6.7, 6
  Tris-citrate, pH 8.0, 6
  Tris-glycine (disc), 6
  Tris versene borate, 6

Computer program
  BIOSYS-1, 7, 23, 31
  Taxan, 7
Contact zones, 52, 53
Cophenetic correlation coefficient, 22, 23
Cylindrosoma glutinosum, 62

Distance
  Build-up of genetic, 12, 50
  Cavalli-Sforza and Edwards, 23
  Edwards, 23
  Immunological, 83–86, 91, 145–149
  Nei standard genetic (*D*), 6, 12, 13, 23, 26–30, 51, 83, 91, 145–149
  Prevosti, 23
  Rogers, 23
Drosophila, 47
Drosophila willistoni group, 47

Electrophoresis, 82
  Acrylamide disc, 6
  Sequential, 90
  Starch gel, 6
Esterase (Est), 6, 9, 39
Evolution
  Differential rates, 39, 42
  Morphological, 12
  Protein, 12

Fumarase (Fum), 6

Gel, 6
  Acrylamide disc, 6
  Starch, 6
Genetic
  Data, 4, 105–144
  Differentiation
    Species level, 13
    Structural gene, 49
  Distance. *See* Distance: Nei standard genetic
  Identity (Nei's *I*), 6–8
  Loci, substitutions within, 39–43
  Variability, 43–45
Glutamate dehydrogenase (Gdh), 6, 9, 13, 42
Glutamic oxaloacetic transaminase (Got), 6, 39
Grouping of populations, 9–23
Groups. *See under species names*

Hennigian methods, 49
Heterozygosity (*H*), 7, 43–45
Hydromantes, 91
  Slope of linear regression between *ID* and *D*, 91

Interpopulation relationships, 31–37
  Pleistocene effects on, 37–39
Isocitrate dehydrogenase (Idh), 6, 9

Lactate dehydrogenase (Ldh), 6, 39
Leucine aminopeptidase (Lap), 6
Localities, list of, 101–104

Malate dehydrogenase (Mdh), 6, 9, 39
Micro-complement fixation (MC'F), 81, 82, 90, 92
Molecular clock, 12, 26
Molecular evolution, 82
  Equal rates of, 14, 22
Monomorphic locus, 4, 6
Morphological evolution
  Convergent, 82
  Relation to molecular evolution, 82

National Museum of Natural History (USNM), 9
Null alleles, 9

Peptidase (Pep), 6, 9
Phenogram, UPGMA, 7–9, 14, 22, 23, 25, 33, 35, 36, 43
Phosphoglucomutase (Pgm), 6
Phosphoglucose isomerase (Pgi), 6
Phylogeny, errors in reconstruction, 20
Pleistocene effects on interpopulation relationships, 26, 34, 37–39
Plethodon
  Previous genetic variation studies, 4
  Life history study, 47
  Slope of linear regression between *ID* and *D*, 91
  Species groups, 12
Plethodon albagula (group 13), 18, 19, 35, 36, 38, 46, 71–73, 78
  Albumin antibody, 83, 84, 87, 88
  Genic variation, 131–134, 137–140
  Heterozygosity estimates, 43, 134, 140
  Immunological distances, 84–86, 88, 91, 145–149
Plethodon aureolus (group 15), 3, 9, 14, 20, 25, 26, 37, 45, 46, 50, 52, 53, 75
  Genic variation, 141–144
  Heterozygosity estimates, 43, 144
  Immunological distances, 86, 88–91, 149
Plethodon caddoensis, 39, 43, 51
Plethodon chattahoochee (group 2), 15, 33, 34, 46, 55–58
  Genic variation, 105–109
  Heterozygosity estimates, 43, 109
  Immunological distances to, 86, 87, 90–92, 145
Plethodon chlorobryonis (group 3), 15, 16, 34, 36, 37, 46, 58, 59
  Albumin antibody, 83–85, 87, 88
  Genic variation, 109–112
  Heterozygosity estimates, 43, 112
  Immunological distances, 84–87, 90–92, 145–149
Plethodon cinereus, 31, 34, 37
Plethodon cylindraceus (group 12), 16, 18, 19, 33, 35, 36, 38, 46, 70–72
  Genic variation, 134–137
  Heterozygosity estimates, 43, 137
  Immunological distances, 86, 88, 90, 91, 148
Plethodon fourchensis, 39, 51
Plethodon glutinosum, 61
Plethodon glutinosus (group 6), 3, 9, 14, 17, 34–36, 38, 46, 52, 54, 61–63, 75, 76
  Albumin antibody, 82–84, 87, 88
  Genic variation, 115–127
  Heterozygosity estimates, 43, 118, 121, 124, 127
  Immunological distances, 84–87, 89–91, 145–149
  Species complex, 3, 7, 10, 12, 15, 16, 31, 42, 43, 45, 50, 51, 53, 54, 76, 82, 84, 92
  Species group, 12, 39, 42, 43, 45
Plethodon glutinosus albagula, 71. See also *Plethodon albagula*
Plethodon glutinosus chlorobryonis, 58. See also *Plethodon chlorobryonis*
Plethodon glutinosus glutinosus, 54, 55, 62. See also *Plethodon glutinosus*
Plethodon glutinosus grobmani, 69. See also *Plethodon grobmani*

Plethodon grobmani (group 11), 18, 35, 36, 39, 46, 69, 70
Genic variation, 131–134
Heterozygosity estimates, 43, 131–134
Immunological distances, 86, 88, 90, 91, 148
Plethodon jordani, 14, 15, 26, 31, 33, 39, 51, 54, 55
Albumin antibody, 83–86
Complex, 7
Immunological distances, 84, 85, 91
Plethodon jordani kentucki, 75
Plethodon jordani rabunensis, 15, 55
Plethodon jordani teyahalee, 54
Plethodon kentucki (group 16), 3, 9, 20, 23, 25, 26, 37, 45, 46, 50, 52, 53, 62, 75, 76
Genic variation, 141–144
Heterozygosity estimates, 43, 144
Immunological distances, 85, 86, 88, 91, 92, 149
Plethodon kiamichi (group 7), 17, 35, 36, 46, 62, 64, 65
Genic variation, 127–131
Heterozygosity estimates, 43, 131
Immunological distances, 86, 87, 90, 91, 147
Plethodon kisatchie (group 9), 18, 35, 36, 38, 46, 67, 68
Genic variation, 131–134
Heterozygosity estimates, 43, 134
Immunological distances, 86, 89, 90, 91, 147, 148
Plethodon mississippi (group 8), 17, 18, 35, 36, 38, 39, 46, 65–67
Genic variation, 127–131
Heterozygosity estimates, 43, 131
Immunological distances, 86, 87, 90, 91, 147
Plethodon ocmulgee (group 5), 16, 17, 34, 35, 39, 42, 46, 60, 61
Genic variation, 112–115
Heterozygosity estimates, 43, 115
Immunological distances, 86, 87, 90, 91, 146
Plethodon ouachitae, 17, 35, 39, 43, 51
Plethodon petraeus, 42
Plethodon punctatus, 39, 43
Plethodon savannah (group 14), 20, 36, 37, 46, 73, 74
Genic variation, 141–144
Heterozygosity estimates, 43, 144
Immunological distances, 88, 90, 91, 149
Plethodon sequoyah (group 10), 18, 35, 36, 46, 68, 69
Genic variation, 131–134
Heterozygosity estimates, 43, 134
Immunological distances, 87, 90, 91, 148
Plethodon serratus, 31
Plethodon shermani, 55
Plethodon shermani rabunensis, 55
Plethodon teyahalee (group 1), 3, 9, 14, 15, 25, 31, 33, 35, 36, 38, 46, 52, 54, 55
Genic variation, 105–109
Heterozygosity estimates, 43, 109
Hybrid origin, 33
Immunological distances, 86, 87, 91, 145
Plethodon variolatus (group 4), 16, 34, 39, 46, 59, 60
Genic variation, 112–115
Heterozygosity estimates, 43, 115
Immunological distances, 86, 87, 90, 91, 146
Plethodon variolosum, 59
Plethodon wehrlei, 39, 43
Group, 39, 43
Plethodon yonahlossee, 39, 51
Populations studied, 4
Selection of, 4
Sites, 5, 101–104
Protein-buffer combinations, 4, 6
Protein-1, 6
Protein-2, 6, 9, 14, 42
Protein-3, 4, 6, 39
Pseudoeurycea, 91
Slope of linear regression between *ID* and *D*, 91

Rana pipiens complex, 53
Relationships among groups, 23–37
Reproductive isolation, 50

Salamandra cylindracea, 16, 18, 70
Salamandra glutinosa, 17, 61
Salamandra melanoleuca, 62
Salamandra variolata, 16, 18, 59
Sample size, 4

Semispecies, 47, 48, 50, 52
Six-phosphoglutonate dehydrogenase (6-Pgd), 6, 39
Size, variation among groups, 45–47
Species, 50–53
  Cryptic, 82
Statistics, goodness of fit, 23, 24, 26, 31, 85
  Slope of linear regression between *ID* and *D*, 91
Subspecies, 47, 50–53
Substitutions within loci, 39–43
Superspecies, 50

Taxonomy, 47–76
Transferrin (Trf), 6, 7, 9, 39
Tree, phylogenetic, 22, 81, 84
  Distance Wagner, 7, 9, 14, 23, 32, 33, 35–37, 43, 83

Variability of populations, 43–45

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ISBN 0-252-06007-5