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## Biochemical genetics and systematics of garter snakes of the *Thamnophis elegans-couchii-ordinoides* complex

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OCCASIONAL PAPERS OF THE MUSEUM OF  
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BATON ROUGE, LOUISIANA

BIOCHEMICAL GENETICS AND SYSTEMATICS OF GARTER  
SNAKES OF THE *THAMNOPHIS ELEGANS-COUCHII-*  
*ORDINOIDES* COMPLEXBy ROBIN LAWSON<sup>1</sup> AND HERBERT C. DESSAUER<sup>2</sup>

The garter snakes (genus *Thamnophis*) are among the most ubiquitous of the reptile fauna of North America. The range of the genus is extensive, from the Northwest Territories in Canada to Costa Rica in Central America and from the Atlantic to the Pacific coasts. All types of habitat have been invaded except the most arid areas of the southwest United States (Ruthven, 1908).

Along the Pacific Coast of the North American continent four species of garter snakes are recognized, each of which is sympatric with at least one of the others over parts of their ranges. Of the four species, the wide ranging *Thamnophis sirtalis* is most distinct. The extent of reproductive isolation and the interrelationships of the other three, *Thamnophis elegans*, *T. couchii*, and *T. ordinoides*, have not been clearly defined.

Many herpetologists have worked with this *elegans-couchii-ordinoides* complex without producing a completely satisfactory assessment of the true affinities of the many forms (see Rossman, 1979). In large measure these affinities have been obscured by the lack of adequate samples from critical areas and by convergence in color patterns.

The most recent overall revision of the complex was presented by Fox (1951). He considered *T. ordinoides* to be a monotypic species (Fox,

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1948), and the remaining members as a "rassenkreis" of subspecies of *T. elegans*. In the latter decision he was defending the arrangement proposed by Fitch (1940), but Fox showed that the subspecies could be assembled into two groups having either aquatic or terrestrial ecological preferences. He observed that the two groups occurred sympatrically over much of their ranges without interbreeding, thus acting as if they were distinct species. Due to apparent intergradation in the northern part of the range, Fox perpetuated Fitch's conclusion that these snakes represent a single species. A semiaquatic, allopatric race of the Klamath Basin, *biscutatus*, was described as intergrading in northeastern California with the terrestrial group through the races *elegans* to the south and *vagrans* to the east. The race *biscutatus* also was believed to intergrade with *hydrophilus* of the aquatic group along the Klamath River drainage in north-central California.

Comparative protein evidence has been a major factor in showing that *Thamnophis elegans* (*sensu* Fox, 1951) should be split into the two species, *T. elegans* and *T. couchii*, recognized by Stebbins (1966). Dessauer, Fox, and Hartwig (1962) found that subspecies of the aquatic and terrestrial groups had different transferrins. Subsequently, Fox and Dessauer collected specimens along the Klamath River drainage where *biscutatus* and *hydrophilus* were presumed to intergrade. As these specimens did not exhibit transferrin phenotypes characteristic of intergrades, Fox and Dessauer (1965) concluded that the aquatic and terrestrial groups were reproductively isolated and represented two species, *T. elegans* and *T. couchii*. This arrangement agreed with the conclusions presented by Rossman (1964), which were based upon the examination of teeth and other morphological characters of the same series of snakes.

Our paper presents evidence concerned with the distribution of transferrins and other proteins of specimens collected from the Klamath River drainage as well as from other areas of the range of the complex. These data on over 800 specimens offer: (a) estimates of genetic variability that characterize populations of the complex; (b) evaluations of degrees of relationship of 11 forms based upon protein phenotypes at 31 presumptive loci; and (c) suggestions concerning the species substructure of the complex.

#### MATERIALS AND METHODS

Eight hundred and twelve snakes, including samples of 11 nominal forms, were collected at 144 sites (Figs. 1 and 2). Brief descriptions of localities of capture and voucher specimen numbers are given in Table 1;

Table 1. Collecting Sites and Voucher Specimens<sup>a</sup>.

**Thamnophis elegans elegans:** (41 specimens). California: Butte Co. CSUH 3915, 4126; LSMUZ 8128, 19284; Calaveras Co. LSMUZ 8133; WF 4768; Lassen Co. CSUH 4078; LSMUZ 8996, 9002-9004, 9006, 9011, 9018, 9019, 9030, 9070, 10458, 36931-36933, 36937, 36938; Plumas Co. LSMUZ 12768, 12774, 12777, 12801, 12803; Shasta Co. CSUH 3912, 3916, 3921, 3964; Siskiyou Co. LSMUZ 9060-9062, 9063, 9064, 9073, 22244, 28934, 34257; WF 6326, 6331; Oregon: Jackson Co. WF 6395, 6396.

**Thamnophis elegans biscutatus:** (134 specimens). California: Lassen Co. CSUH 3888, 3889, 3923; LSMUZ 19119, 19123, 19302, 20295-20297, 21190, 22156, 22210, 28937; WF 6097, 6100, 6106; Modoc Co. LSMUZ 14051-14055, 14057-14062, 19295, 19298, 21244-21248, 22188, 22200-22202, 22204, 34232; WF 6234, 6247, 6262, 6271, 6272, 6327-6329, 6335; Oregon: Klamath Co. CSUH 3797, 3809, 3895, 3950, 3978, 4098-4101; LSMUZ 8533, 8534, 9053-9059, 9066-9068, 9092, 10424, 10456, 12770, 12793-12797, 19121, 19125, 19127-19130; 19151, 19154-19155, 19299-19300, 22203, 22211, 22212, 22229, 28915, 28936, 34233, 34258; WF 6095, 6346, 6348b, 6350, 6352, 6372, 6374, 6556; Lake Co. LSMUZ 9069, 9114, 10488, 19166, 19292-19294, 19296, 19297, 20360, 22114-22116, 22135-22143; WF 6591-6593.

**Thamnophis elegans terrestris:** (126 specimens). California: Alameda Co. LSMUZ 7906, 7908, 7909; WF 4571; Del Norte Co. LSMUZ 8119, 22230-22243, 34273-34275; WF 6504-6508, 6511, 6513-6517, 6518a, b, c; Humboldt Co. CSUH 3891, 3892, 3894, 3902, 3943, 4077, 4087, 4114, 4182, 4183; Marin Co. LSMUZ 7914, 7915; Mendocino Co. CSUH 3799, 3807, 3808, 3881, 3893, 3910, 3914, 3949, 3969, 3970, 4070, 4119; Monterey Co. CSUH 3883-3888; San Mateo Co. CSUH 3760, 3761, 3767, 3770, 3775, 3905, 3958-3960, 3996-3998, 4003, 4011-4014, 4016, 4017, 4069, 4102, 4131-4140, 4143, 4144, 4146-4148, 4150, 4155, 4156, 4158, 4159; LSMUZ 7910-7912, 7916, 7917, 7920, 19285-19291, 20356-20358; WF 3556, 3557.

**Thamnophis elegans vagrans:** (90 specimens). Colorado: Archuleta Co. CSUH 3777; Alamosa Co. LSMUZ 9076-9088, 10616-10619, 12786, 12789, 12805-12810; La Plata Co. CSUH 3928-3932, 3938, 3946, 3951-3957, 3972-3977, 3991, 3992, 3995, 4061-4067, 4081-4084, 4088-4092; New Mexico: Rio Arriba Co. CSUH 3983-3986, 3991, 3994, 4044, 4045, 4068; LSMUZ 36934; Taos Co. LSMUZ 36935; Utah: Utah Co. LSMUZ 12827; Washington: Snohomish Co. LSMUZ 8004-8006; Spokane Co. LSMUZ 8282; WF 5086, 5088-5090; Thurston Co. LSMUZ 8283; Wyoming: Teton Co. WF 4407.

**Thamnophis couchii couchii:** (73 specimens). California: Butte Co. CSUH 3762, 3802-3804, 4202, 4203; Placer Co. LSMUZ 22123, 22124; Plumas Co. CSUH 3768, 3769, 3776, 3809, 4072-4074, 4120, 4121, 4129, 4130; LSMUZ 9013, 9015, 9022-9025, 9027-9029, 10465; WF 6620; Lassen Co. LSMUZ 8997, 9001, 9005, 9014, 9016; Shasta Co. CSUH 3806; LSMUZ 9000, 9007, 9017, 9021, 9075, 9115-9116, 10332, 20355, 22209, 34567-34590, 35178, 35179, 36718; HD 2653; Tehama Co. LSMUZ 34261-34267, 36671, 36673; WF 6300a; HD 2656; Tulare Co. LSMUZ 8993, 8998, 9012, 10470; Tuolumne Co. LSMUZ 34584, 34585; Nevada: Washoe Co. LSMUZ 19116, 19117.

**Thamnophis couchii hammondi:** (51 specimens). California: Los Angeles Co. WF 5983; San Diego Co. CSUH 3798, 3906, 3911, 3920, 3925, 3927, 3968, 3971, 4033, 4034, 4041, 4096; LSMUZ 7919, 9010, 9011, 9031-9037, 9108-9110, 9020, 9026, 9093, 10349-10353, 14680, 14681; WF 4940, 5970, 5983; San Luis Obispo Co. CSUH 4013; LSMUZ 23894, 23895; HD 1356; Unknown Co. LSMUZ 7924, 8763, 8767; WF 5418, 5420, 5422.

**Thamnophis couchii atratus:** (57 specimens). California: Alameda Co. LSMUZ 8129, 34591-34593; Contra Costa Co. LSMUZ 8131; Monterey Co. CSUH 4037, 4093, 4179; San Francisco Co. LSMUZ 8305, 8306; San Mateo Co. CSUH 3773, 3774, 3937, 3962-3963, 3989, 4005-4010, 4020-4023, 4141, 4142, 4145-4147, 4151, 4153, 4154, 4157, 4171-4176, 4200, 4201; LSMUZ 24352-24356, 20359, 28911-28914, 28931-28933, 34255.

**Thamnophis couchii aquaticus:** (28 specimens). California: Glenn Co. WF 6321, 6322; Marin Co. LSMUZ 8220-8227, 22205-22208; WF 4575, 6459a; Mendocino Co. CSUH 3790-3793, 3882, 3948; HD 2661; Sonoma Co. CSUH 3896; LSMUZ 34582, 34583.

**Thamnophis couchii gigas:** (21 specimens). California: Butte Co. LSMUZ 8066-8069, 8532, 9090, 9091, 9120, 10341, 10343, 10464, 10483, 10518, 14050, 16926, 20562, 20845, 20943, 21080; Merced Co. LSMUZ 22155; San Joaquin Co. LSMUZ 35176.

**Thamnophis couchii hydrophilus:** (116 specimens). California: Humboldt Co. LSMUZ 22245, 22246, 34572-34581; Mendocino Co. LSMUZ 8130, 36754-36759; HD 2652; WF 4670; Shasta Co. LSMUZ 35177, 36690, 36695, 36705, 36747-36753; HD 2654, 2655; WF 6324; Siskiyou Co. LSMUZ 9038-9046, 9048-9050, 9074, 16818, 19110-19112, 19126, 19152, 22192, 28935, 28938-28951, 34279, 36414; WF 6413, 6428; Trinity Co. LSMUZ 34594-34601; Oregon: Jackson Co. LSMUZ 20929, 21092, 22120, 22189-22191, 22193-22199; WF 6390-6394, 6526-6528, 6550; CSUH 3800, 3801, 3907, 3913, 3926, 4043, 4097, 4180, 4181; Josephine Co. CSUH 3979, 4000, 4053, 4085, 4086, 4170.

**Thamnophis ordinoides:** (75 specimens). British Columbia: Vancouver Island CSUH 3781, 3789, 3796, 3980, 3981, 4024, 4025, 4040, 4052, 4057-4062, 4161, 4162; California: Del Norte Co. LSMUZ 19118, 34229, 34230, 34252, 34253, 34268-34272; WF 6472, 6473, 6475, 6480, 6519; Unknown Co. WF 3749-3751, 3753; Oregon: Curry Co. CSUH 3779, 3987, 4001, 4002, 4026-4032, 4036, 4038, 4039, 4048, 4050, 4051, 4094-4095, 4178; Multnomah Co. LSMUZ 12811-12822; Washington: Gray's Harbor Co. LSMUZ 8299; Pierce Co. LSMUZ 8297, 8298; Thurston Co. LSMUZ 8300, 8301; Snohomish Co. LSMUZ 8007, WF 3907.

<sup>a</sup>Voucher specimens maintained at: CSUH - California State University at Hayward; LSMUZ - Louisiana State University Museum of Zoology; HD and WF - preliminary catalog numbers of voucher specimens utilized for biochemical studies that will be cataloged into the LSMUZ collection.

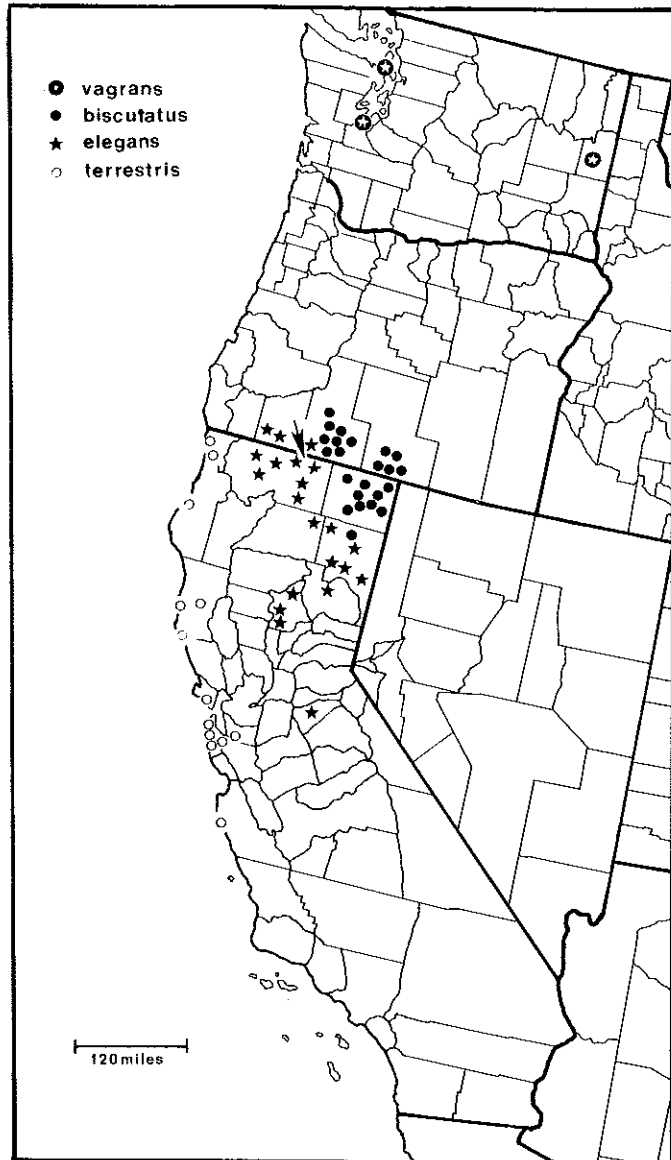


FIGURE 1. Distribution of population samples of *Thamnophis elegans* (terrestrial group of Fox, 1951) used in this study. Solid arrow points to the area where Fitch (1940) and Fox (1951) believed that populations of the terrestrial and aquatic groups intergrade.

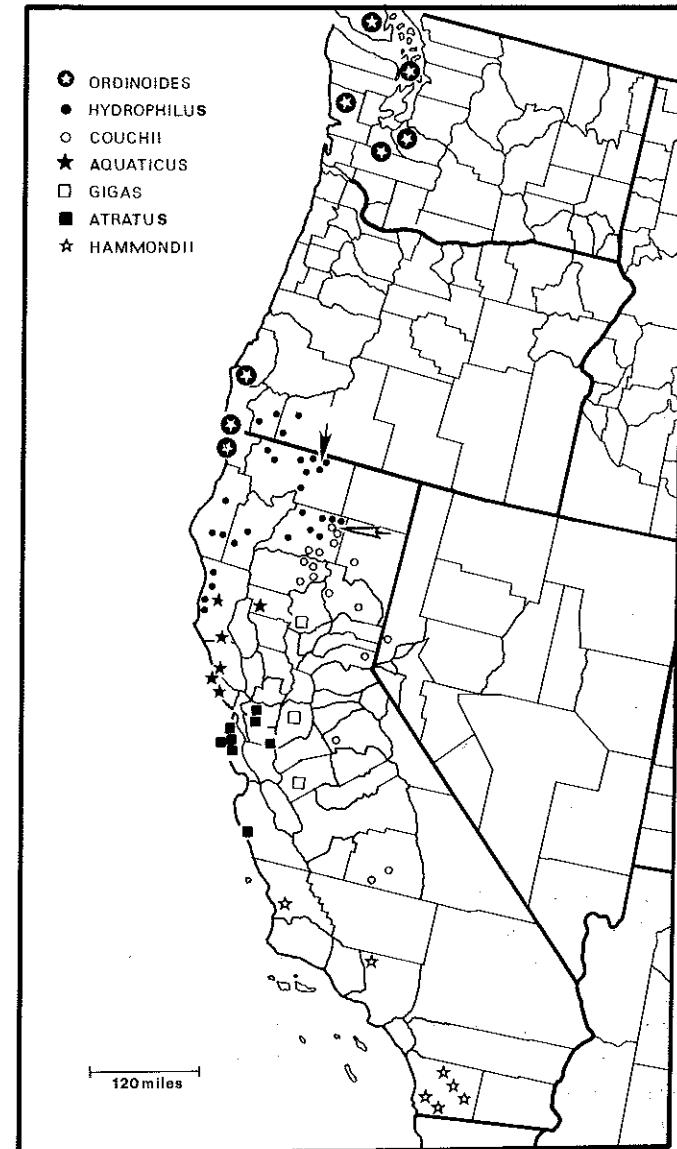


FIGURE 2. Distribution of population samples of *Thamnophis ordinoides* and of *Thamnophis couchii* (aquatic group of Fox, 1951) used in this study. Solid arrow points to the area where Fitch (1940) and Fox (1951) believed that populations of the terrestrial and aquatic groups intergrade. Open arrow points to the region where the ranges of *couchii* and *hydrophilus* come into contact.

more exact locality data are recorded with the preserved or skeletonized specimens. These are deposited either at the Louisiana State University Museum of Zoology in Baton Rouge (LSUMZ) or the Zoological Museum of California State University at Hayward (CSUH).

Snakes were collected during the past 20 years by ourselves and by other scientists, principally by our deceased colleague Dr. Wade Fox, and by Drs. Douglas A. Rossman of Louisiana State University and Glenn R. Stewart of California State Polytechnic University-Pomona. Rossman has utilized many of these specimens in his morphometric study (Rossman, 1979), and he and Stewart are using the remainder in their investigation. Many of these snakes were captured during the summer of 1963 on field trips sponsored by the American Philosophical Society. The majority of the remaining specimens were collected during 1977 and 1978.

Snakes were anesthetized, using ether, chloroform or pentobarbital, and opened ventrally. Blood was collected in a heparinized syringe either from a cardiac puncture or, in the case of small animals, from the severed dorsal aorta or vena cava. Only blood was sampled from animals acquired before 1968. From more recently collected specimens samples of heart, liver, kidney, and skeletal muscle also were removed, wrapped in aluminum foil, and quick frozen on a block of dry ice. Plasma and red cells were separated and stored with the other tissues in a freezer at  $-20^{\circ}\text{C}$  or lower until needed. Many analyses were performed on the day of tissue collection with most testing completed within 2 months. Some of the proteins analyzed were very stable in the frozen state. For example, neither the electrophoretic patterns nor the immunological properties of plasma transferrin were altered after 20 years in storage (Mao and Dessauer, 1971). Samples of tissues unused during this study are being maintained in the frozen collection of herpetological tissues in the Biochemistry Department at Louisiana State University Medical Center in New Orleans.

In the preparation of tissues for enzyme analysis, red cells were hemolyzed in one or two volumes of water and centrifuged at 5,000 g or higher to remove cell debris. Samples of frozen tissues were minced, mixed with one or two volumes of 0.25 M sucrose, hand homogenized in a glass unit, and centrifuged at at least 5,000 g to remove cell debris. All such operations were carried out in the cold.

Proteins in the supernatant solutions of hemolysates and homogenates, and in blood plasma, were analyzed by either vertical (Smithies, 1959) or horizontal (Ayala *et al.*, 1972) starch-gel electrophoresis. Tris-hydroxymethane (TRIS), citric and boric acid were the principle constituents of the four buffers (Table 2). Horizontal gels were cooled with packets of

Blue Ice (Divajex Co., Tustin, California); vertical-gel electrophoresis was carried out in a cold room maintained at about  $4^{\circ}\text{C}$ .

Proteins were localized on gel slices using either specific stains or autoradiography. Staining techniques for the majority of enzymes closely followed methods described by Harris and Hopkinson (1976). In addition, octanol dehydrogenase was localized by the method of Courtright *et al.* (1966) and glutamate dehydrogenase by the method of Brewer (1970). Transferrins were identified either in the rivanol-soluble fraction of plasma (Matthews, 1975) or by iron-59 binding and autoradiography (Giblett *et al.*, 1959). Activities designated as aconitases were observed in gels made in citrate buffers treated with a developing solution containing nicotinamide adenine dinucleotide phosphate, tetrazolium, and phenazine methosulfate (Harris and Hopkinson, 1976). Proteins from different individuals along with standards were compared side by side on the same gel to avoid errors inherent in comparisons based upon relative mobilities. Albumin and transferrin and two additional nonenzymic proteins were analyzed in plasma; 6-phosphogluconate dehydrogenase and, for some specimens, superoxide dismutase were analyzed in hemolysates. All other enzymes were analyzed in mixed homogenates of liver and heart muscle. Transferrin, superoxide dismutase, and albumin phenotypes were determined for all specimens, 6-phosphogluconate dehydrogenase for those collected prior to 1968, and all other proteins for specimens collected after 1968.

Frequencies of alleles at 31 presumptive loci were used to calculate a matrix of pairwise Nei genetic distances (Nei, 1972) between members of the complex (Table 5). The method of Fitch and Margoliash (1967), appropriate for evaluating electrophoretic data (Prager and Wilson, 1978), was used to construct the phenogram (Fig. 4) expressing the pattern of divergence suggested by these genetic distances. Four alternative phenograms of slightly different branching orders were developed, but Figure 4 was selected because it best fit the data as judged by the F-value of Prager and Wilson (1978).

#### ELECTROPHORETIC PHENOTYPES OF PROTEINS

Table 2 summarizes information concerning the 19 proteins studied. For those with phenotypes determined by 2 or more presumptive loci (e.g., Ldh), loci are labeled numerically in order of decreasing anodal migration of their products. Alleles at a specific locus are indicated with lower case letters and are labeled alphabetically beginning with the product migrating farthest toward the anode. Identifications concerning the 31 presump-

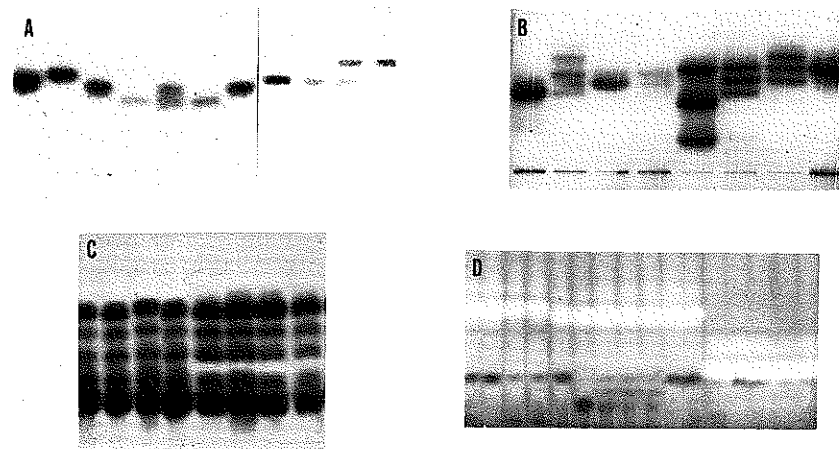


FIGURE 3. Phenotypes of polymorphic proteins of the *elegans-couchii-ordinoides* complex. Presumed genotypes for each protein read from left to right: (A) Transferrin: d/e, d/d, e/e, f/f, e/f, f/f, e/e, d/d, d/d, b/d, b/b; (B) 6-phosphoglucuronate dehydrogenase: e/e, a/e, d/d, c/e, b/f, b/e, a/d, b/b; (C) Lactate dehydrogenase: Ldh-1 are all b/b; Ldh-2, 4 type c/c followed by 4 type d/d; (D) Octanol dehydrogenase (dark bands): 5 type b/b, c/c, 3 b/c, and 8 b/b; Superoxide dismutase (light bands): 5 type a/a, 7 type b/b, and 5 type c/c.

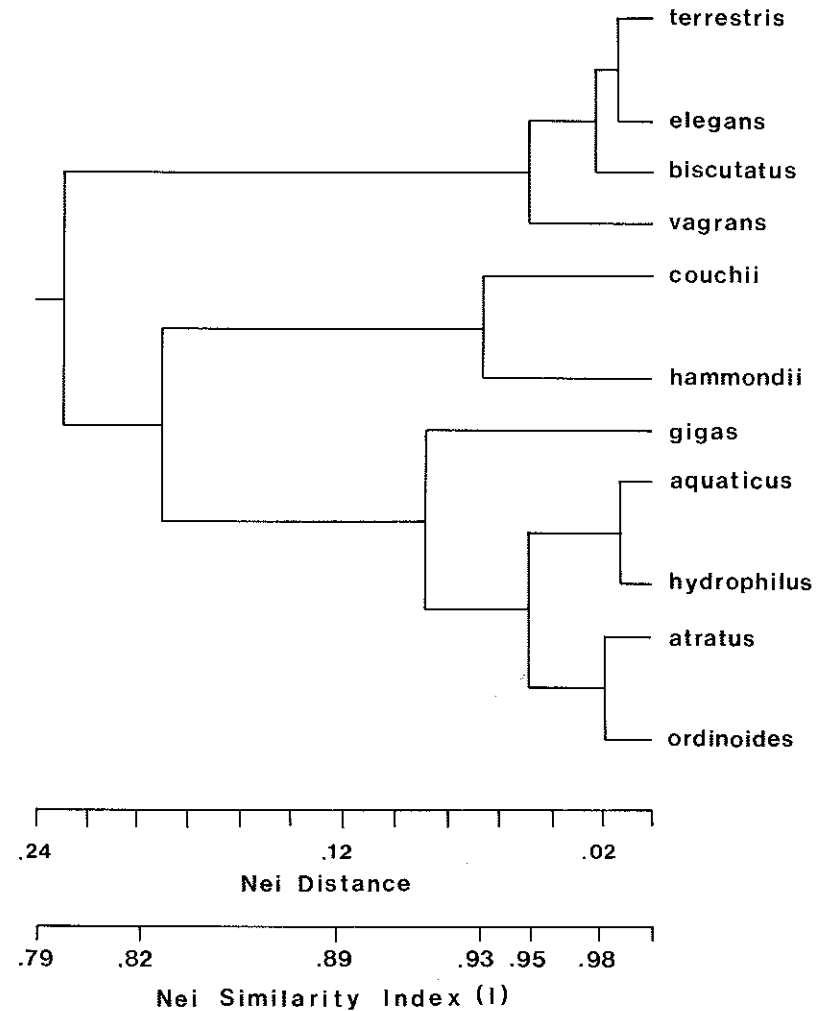


FIGURE 4. Phenogram of genetic affinities of members of the *elegans-couchii-ordinoides* complex as suggested by the protein evidence. Branching sequence was developed from the matrix of Nei distances (Table 6) using the method of Fitch and Margoliash (1967). Nei similarity index ( $I = -\log_e D$ ) is an estimate of the proportion of genes common to divergent populations (Nei, 1972).

tive loci are based upon specificities of staining reactions, tissue differences in electrophoretic phenotypes, and on phenotypes for the different proteins observed in organisms for which breeding evidence is available (Manwell and Baker, 1970; Ward, 1977; Harris and Hopkinson, 1976; Dessauer and Zweifel, unpubl. data). Generally the tissue in which these different loci were active and the banding observed for homozygous and heterozygous individuals were similar to those previously described for *Thamnophis* (Gartside *et al.*, 1977) and for many other vertebrates. For example, transferrin of the plasma migrated as a single subunit protein, with homozygotes exhibiting single-banded, and heterozygotes double-banded, patterns (Fig. 3a). Red cell 6-phosphogluconate dehydrogenase acted as a 2 subunit protein with homozygotes exhibiting single-banded and heterozygotes triple-banded patterns (Fig. 3b). In tissues in which both heart type (Ldh-1) and muscle type (Ldh-2) lactate dehydrogenases were active, electrophoretic phenotypes suggested that polypeptide subunits of Ldh associate into 5 tetrameric isozymes. As is true for the Ldh's of certain primates (Koen and Goodman, 1969), homotetramers of Ldh-2 locus of some tissues had identical mobilities but the pattern of intermediate bands was distinctive for different alleles (Fig. 3c). Except for phosphoglucose isomerase, all proteins migrated toward the anode in the buffers used.

#### PROTEIN POLYMORPHISM THROUGHOUT THE COMPLEX

Table 3 presents gene frequencies for the 13 loci that were polymorphic at the 5% level in one or more individual population samples. Of the 18 remaining loci, 14 were monomorphic and 4 exhibited only rare variants (Table 2). Mean heterozygosities across the complex exceeded 0.1 for Pgm-2, Pep-2, Pgd, Trf, and Odh-3. Phosphoglucose mutase-2 was the most highly polymorphic locus, having a mean heterozygosity of 0.445 across the complex and exceeding 0.6 in the samples of *T. e. elegans*, *T. c. hammondi*, *T. c. atratus*, and *T. c. hydrophilus* (Table 4).

Far more genetic variation at the protein level was present in the complex than was distributed among individual populations. Of the 82 alleles detected at the 31 loci (Table 2), an average of 47 (40 to 53) were found in individual species, and an average of 40 (34 to 46) in individual subspecies (Table 3). Alleles at 5 loci (Ldh-2, Sod, Trf, Act-2, and Got-1) were restricted largely to *T. elegans*, to *T. ordinoides*, or to one or the other of the divergent subgroups within *T. couchii* that will be described subsequently. Nevo (1978), in a review of genetic variation in natural popula-

Table 2. Proteins Analyzed and Loci Scored.

| Protein                             | Buffers(s) <sup>a</sup> | Tissue(s) <sup>b</sup> with Highest Activity | Loci Scored | Alleles detected Across the Complex |
|-------------------------------------|-------------------------|--|-------------|-------------------------------------|
| OXIDOREDUCTASES                     |                         |  |             |                                     |
| glycerol-3-phosphate dehydrogenase  | 1                       | L, M   | Gpd-1       | 1                                   |
| lactate dehydrogenase               | 1                       | H, K, R                                      | Gpd-2       | 1                                   |
|                                     |                         | M, L   | Ldh-1       | 2 <sup>c</sup>                      |
|                                     |                         | H, K, L                                      | Ldh-2       | 6                                   |
| malate dehydrogenase                | 3                       | H, K, L                                      | Mdh-1       | 1 <sup>d</sup>                      |
|                                     |                         | H, K, L                                      | Mdh-2       | 2 <sup>d</sup>                      |
| malate enzyme                       | 3                       | H  | Me          | 1                                   |
| 6-phosphogluconate dehydrogenase    | 4                       | R  | Pgd         | 6                                   |
| octanol dehydrogenase               | 1                       | L  | Odh-1       | 1                                   |
|                                     |                         |  | Odh-3       | 3                                   |
| superoxide dismutase                | 1, 4                    | L, H, R                                      | Sod         | 3                                   |
| glutamate dehydrogenase             | 1                       | L  | Gdh         | 1                                   |
| TRANSFERASES                        |                         |  |             |                                     |
| glutamate oxaloacetate transaminase | 1                       | H, K, L                                      | Got-1       | 4                                   |
| phosphoglucose mutase               | 1                       | H, K, L                                      | Pgm-1       | 2                                   |
| LYASES                              |                         |  |             |                                     |
| aconitase                           | 3                       | H, K, L                                      | Pgm-2       | 5                                   |
|                                     |                         | L  | Act-1       | 2 <sup>a</sup>                      |
|                                     |                         |  | Act-2       | 3                                   |
| HYDROLASES                          |                         |  |             |                                     |
| esterases                           | 2                       | L, P   | Est-1       | 2                                   |
|                                     |                         | L  | Est-3       | 1                                   |
| alkaline phosphatases               | 1                       | L, P   | Akp-1       | 1                                   |
|                                     |                         | L  | Akp-2       | 1                                   |
|                                     |                         | L  | Akp-3       | 1                                   |
| tripeptidase (leu • gly • gly)      | 2                       | L, H   | Pep-1       | 3                                   |
|                                     |                         | L, H   | Pep-2       | 6                                   |
| leucine amino peptidase             | 2, 5                    | L  | Lap-1       | 1                                   |
|                                     |                         | P  | Lap-2       | 3 <sup>f</sup>                      |
| ISOMERASES                          |                         |  |             |                                     |
| Phosphoglucose isomerase            | 2                       | L  | Pgi-1       | 5                                   |
| NONENZYMIC PROTEINS                 |                         |  |             |                                     |
| albumin                             | 1, 5                    | P  | Alb         | 1                                   |
| transferrin                         | 1, 5                    | P  | Trf         | 6                                   |
| plasma protein                      | 1, 5                    | P  | Pp-1        | 1                                   |
|                                     |                         | P  | Pp-2        | 1                                   |

<sup>a</sup>Buffer 1: TRIS-citrate, pH 8.6 (Poulik, 1957); buffer 2: TRIS-citrate, lithium borate, pH 8.2 (Sefander *et al.* 1971); buffer 3: TRIS-citrate pH 7 (Ayala *et al.* 1972); buffer 4: TRIS-EDTA-borate, pH 8.1 (Huisman, 1969); buffer 5: borate, pH 8.6 (Smithies, 1959).

<sup>b</sup>P=plasma, R = red blood cells, L = liver, M = skeletal muscle, H = heart, K = kidney

<sup>c</sup>One heterozygous specimen of *terrestris* exhibited a variant fast allele.

<sup>d</sup>One heterozygous specimen of *biscutatus* exhibited a variant slow allele.

<sup>e</sup>Three heterozygous specimens of *hydrophilus* exhibited variant fast alleles.

<sup>f</sup>One heterozygous specimen of *gigas* exhibited a variant slow allele; one heterozygous specimen of *elegans* exhibited a variant fast allele.





Table 3. (continued)

| Taxon                    | N   | a    | b     | c    | d     | e    | f    | n |
|--------------------------|-----|------|-------|------|-------|------|------|---|
| Transferrin              |     |      |       |      |       |      |      |   |
| <i>T. e. elegans</i>     | 40  |      | 1.000 |      |       |      |      |   |
| <i>T. e. biscutatus</i>  | 140 |      | 1.000 |      |       |      |      |   |
| <i>T. e. terrestris</i>  | 104 |      | .985  |      |       |      |      |   |
| <i>T. e. vagrans</i>     | 70  | .014 | .986  |      | .015  |      |      |   |
| <i>T. e. couchii</i>     | 66  |      |       |      | .015  | .583 | .402 |   |
| <i>T. e. hammondii</i>   | 51  |      |       |      | .981  | .009 |      |   |
| <i>T. e. atratus</i>     | 31  |      | .016  |      | .710  | .161 | .113 |   |
| <i>T. e. aquaticus</i>   | 26  |      |       |      | 1.000 |      |      |   |
| <i>T. e. gigas</i>       | 18  |      |       |      | 1.000 |      |      |   |
| <i>T. e. hydrophilus</i> | 110 |      | .023  |      | .989  | .004 | .004 |   |
| <i>T. e. ordinoideus</i> | 65  |      |       | .177 | .790  | .062 | .062 |   |

Table 4. Proportions of Heterozygotes at Polymorphic Loci.

|                          | Ldh-2 | Pgd  | Odh-3 | Sod  | Got-1 | Pgm-1 | Pgm-2 | Act-2 | Est-1 | Pep-1 | Pep-2 | Pgl-1 | Trf  | Mean for taxon <sup>a</sup> |
|--------------------------|-------|------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|------|-----------------------------|
| <i>T. e. elegans</i>     | .000  | .174 | .385  | .000 | .000  | .000  | .615  | .077  | .000  | .000  | .385  | .000  | .000 | .050                        |
| <i>T. e. biscutatus</i>  | .000  | .273 | .417  | .000 | .000  | .000  | .250  | .000  | .000  | .000  | .167  | .000  | .000 | .021                        |
| <i>T. e. terrestris</i>  | .279  | .210 | .088  | .000 | .000  | .000  | .441  | .000  | .000  | .000  | .353  | .030  | .009 | .045                        |
| <i>T. e. vagrans</i>     | .167  | .298 | .000  | .000 | .000  | .000  | .019  | .000  | .241  | .000  | .000  | .000  | .029 | .024                        |
| <i>T. e. couchii</i>     | .000  | .188 | .000  | .000 | .000  | .174  | .261  | .000  | .000  | .000  | .000  | .043  | .258 | .030                        |
| <i>T. e. hammondii</i>   | .000  | .000 | .000  | .000 | .000  | .000  | .615  | .000  | .000  | .000  | .000  | .308  | .020 | .031                        |
| <i>T. e. atratus</i>     | .000  | .286 | .000  | .000 | .026  | .000  | .615  | .000  | .000  | .436  | .795  | .000  | .469 | .065                        |
| <i>T. e. aquaticus</i>   | .000  | .560 | .000  | .000 | .000  | .167  | .500  | .000  | .000  | .000  | .500  | .000  | .000 | .054                        |
| <i>T. e. gigas</i>       |       | .393 |       |      |       |       |       |       |       |       |       |       |      |                             |
| <i>T. e. hydrophilus</i> | .067  | .553 | .000  | .096 | .087  | .000  | .652  | .266  | .000  | .000  | .227  | .000  | .000 | .065                        |
| <i>T. e. ordinoideus</i> | .091  | .000 | .256  | .159 | .000  | .477  | .477  | .477  | .000  | .273  | .477  | .000  | .308 | .067                        |
| Mean for locus           | .062  | .257 | .115  | .023 | .011  | .036  | .445  | .036  | .024  | .071  | .290  | .038  | .103 |                             |

<sup>a</sup>Sum of heterozygotes/31<sup>b</sup>Blanks = only 1 specimen available

tions, noted that such distribution of variation may be an important general adaptive pattern.

Mean heterozygosity ( $\bar{H}$ ) across the complex as a whole equaled 0.047. Among individual putative species,  $\bar{H}$  was lowest for *T. elegans* (0.031) and for the *couchii* subgroup of *T. couchii* (0.031) and highest for *T. ordinoides* (0.067) and the *atratus* subgroup of *T. couchii* (0.068). Mean heterozygosities for the different subspecies ranged from a minimum of 0.021 for the sample of *T. e. biscutatus* to 0.085 for *T. c. atratus* (Table 4). For individual populations from which 8 or more specimens were analyzed, mean heterozygosities ranged from 0.016 for a sample of *T. c. hammondii* from San Diego County, California, to a high of 0.082 for a sample of *T. c. atratus* from San Mateo County, California (Table 5). These levels of polymorphism are similar to those characterizing populations of *T. sauritus* and *T. proximus* from eastern United States (Gartside *et al.*, 1977).

Table 5. Mean heterozygosity  $\bar{H}^a$ , and percent of loci polymorphic P for selected populations<sup>b</sup>.

|                          |  | N  | $\bar{H}$ | P    |
|--------------------------|--|----|-----------|------|
| <i>T. e. terrestris</i>  | Samoa Peninsula<br>Humboldt Co. CA               | 10 | .030      | 12.9 |
| <i>T. e. biscutatus</i>  | Klamath River<br>Klamath Co. OR                  | 9  | .028      | 12.9 |
| <i>T. c. couchii</i>     | N. Fork Feather River<br>Butte and Plumas Co. CA | 13 | .034      | 12.9 |
| <i>T. c. hammondii</i>   | Picnic Lake Park, Potrero<br>San Diego Co. CA    | 8  | .016      | 6.4  |
| <i>T. c. atratus</i>     | Isenberg Ranch<br>San Mateo Co. CA               | 38 | .082      | 16.1 |
| <i>T. c. hydrophilus</i> | Applegate River<br>Jackson and Josephine Co. OR  | 14 | .078      | 22.6 |

<sup>a</sup>Over 31 loci

<sup>b</sup>Pgd data from same area but may not be derived from the same deme

Table 6. Nei Distances Based on Gene Frequencies at 31 loci.

|                          | T.e.e. | T.e.b. | T.e.t. | T.e.v. | T.e.c. | T.e.h. | T.e.at. | T.e.aq. | T.e.g. | T.e.hy. |
|--------------------------|--------|--------|--------|--------|--------|--------|---------|---------|--------|---------|
| <i>T. e. biscutatus</i>  | .019   |        |        |        |        |        |         |         |        |         |
| <i>T. e. terrestris</i>  | .014   | .022   |        |        |        |        |         |         |        |         |
| <i>T. e. vagrans</i>     | .065   | .037   | .052   |        |        |        |         |         |        |         |
| <i>T. c. couchii</i>     | .228   | .208   | .210   | .231   |        |        |         |         |        |         |
| <i>T. c. hammondii</i>   | .226   | .191   | .219   | .187   | .066   |        |         |         |        |         |
| <i>T. c. atratus</i>     | .243   | .213   | .216   | .214   | .191   | .156   |         |         |        |         |
| <i>T. c. aquaticus</i>   | .259   | .239   | .231   | .276   | .146   | .144   | .034    |         |        |         |
| <i>T. c. gigas</i>       | .273   | .249   | .252   | .258   | .258   | .202   | .070    | .090    |        |         |
| <i>T. c. hydrophilus</i> | .266   | .241   | .232   | .213   | .140   | .108   | .052    | .011    | .092   |         |
| <i>T. ordinoides</i>     | .233   | .207   | .227   | .213   | .180   | .127   | .017    | .030    | .091   | .073    |

#### PROTEIN DIVERGENCE AND RELATIONSHIPS WITHIN THE COMPLEX

Figure 4 is a phenogram expressing degrees of relationship between named members of the complex. It was constructed from a matrix of Nei distances (Table 6) based upon frequencies of alleles at the 31 presumptive protein loci. This phenogram partitions 11 forms of the complex into three divergent groups: (1) subspecies of *Thamnophis elegans*; (2) the subspecies *couchii* and *hammondii* of *T. couchii*; and (3) *atratus*, *aquaticus*, *gigas*, and *hydrophilus* of *T. couchii*, plus *Thamnophis ordinoides*. The phenogram and details regarding the expression of specific alleles throughout the complex will be discussed in terms of: (a) support for species status for *T. elegans*; (b) divergence within *T. couchii*; and (c) the close affinities of *T. ordinoides* and certain subspecies of *T. couchii*.

(a) Support for Species Status for *Thamnophis elegans*.

The subspecies *elegans*, *biscutatus*, *terrestris*, and *vagrans*, are very closely related. These four subspecies cluster together on the phenogram (Fig. 4), as well as on four alternative phenograms developed from the electrophoretic data. Pairwise genetic distances between individual members of the cluster range from a minimum of 0.014 for *terrestris* X *elegans* to a maximum of 0.065 for *elegans* X *vagrans* (Table 6).

These subspecies of *T. elegans* diverge widely from all populations of *T. couchii* and *T. ordinoides*. An average Nei distance of about 0.21 (0.19 to 0.28) separates them from the branch of the phenogram leading to these other members of the complex (Table 6). These relatively high genetic distances arise primarily from contributions of Got-1<sup>e</sup> and Act-2<sup>d</sup>, alleles that were found only in *T. elegans*, and from Sod<sup>b</sup> and Trf<sup>b</sup>, alleles found only rarely in other specimens of the complex (Table 3; also Bellemin and Stewart, 1977). Transferrin and superoxide dismutase were examined in virtually all specimens (Table 1), and these were collected at a wide variety of sites across the western states where the ranges of the subspecies of the two putative species overlap. Only 3 specimens of *T. couchii* (*hydrophilus*, LSUMZ 22199 and 22246; *atratus*, LSUMZ 34255) exhibited heterozygous genotypes (Trf<sup>b</sup>/Trf<sup>d</sup>) expected of hybrids between *T. couchii* and *T. elegans*. Similarly, only one snake having a *T. elegans* morphology (*terrestris*, LSUMZ 19287) exhibited the Trf<sup>d</sup> allele typical of aquatic forms. All *T. elegans* were homozygous for Sod<sup>b</sup>; 7 *T. c. hydrophilus* and 9 specimens of *T. ordinoides* had heterozygous genotypes in which Sod<sup>b</sup> was expressed.

All specimens collected along the Klamath River from Horse Creek to Hornbrook (Fig. 1 and 2, solid arrows), where Fitch (1948) and Fox (1951) believed that *biscutatus* and *hydrophilus* intergrade, had the trans-

ferrin and superoxide dismutase phenotypes typical of their taxon. All *T. e. biscutatus* (the subspecies believed to be the linking form) and *T. e. elegans* had homozygous Trf<sup>b</sup> and Sod<sup>b</sup> genotypes, respectively. Likewise, all specimens of *T. c. hydrophilus* were characterized by homozygous Trf<sup>d</sup> and Sod<sup>c</sup> genotypes. One specimen (LSUMZ 22246) collected near Willow Creek, which lies west of the region of supposed intergradation, has a hybrid transferrin genotype (Trf<sup>b</sup>/Trf<sup>d</sup>), but its Sod phenotype and morphology are typical of *hydrophilus*. The other specimens that exhibit hybrid genotypes at the Trf or Sod loci were collected at scattered sites throughout the geographic range of the complex. Their occurrence suggests that matings may be possible between snakes of the two ecological groups and possibly does occur on rare occasions.

(b) Divergence within *Thamnophis couchii*

Those populations currently named *T. c. atratus*, *T. c. aquaticus*, *T. c. gigas*, and *T. c. hydrophilus* (*atratus* subgroup) cluster together. The average Nei distance between them was 0.058 (0.034 to 0.092; Table 6). Divergence was attributable to small gene frequency differences at a number of loci, especially peptidases (Table 3). The relatively high Nei distance of *T. c. gigas* from other members of the *atratus* subgroup probably reflects the lack of extensive data for *gigas* on all but 3 of the polymorphic loci (Table 3). Similarly, populations designated as *T. c. couchii* and *T. c. hammondii* (*couchii* subgroup) also appear to be close relatives. The Nei distance of 0.066 (Table 6) separating them was attributable to frequency differences at the Pgd, Pgm-2, and Trf loci (Table 3). These differences are large enough to test whether or not the subspecies intergrade, if specimens can be obtained from the Tehachapi Mountains where forms of intermediate morphology have been described (Fitch, 1948).

Although the subspecies within each subgroup appear to be close relatives, the subgroups themselves diverge markedly. The Nei distance separating them averages 0.161 (0.108 to 0.258; Table 6) and was due largely to fixed or nearly fixed alleles at the Ldh-2, Act-2, and Sod loci (Table 3). For example, Sod<sup>a</sup> characterized all specimens of *couchii* and *hammondii* but was not found in any other snake in the complex. The relatively high Nei distance and the fixed alleles that distinguish our samples suggest that the *atratus* and the *couchii* subgroups are either approaching species status or have already attained it. The central question is whether or not populations of the two subgroups intergrade in areas of sympatry or parapatry.

Evidence is available on populations in two such areas of sympatry. In southwestern California, where the ranges of *hammondii* (*couchii* subgroup)

and *atratus* (*atratus* subgroup) overlap extensively, Fox (1951) reported that intergradation does not occur. We found no biochemical evidence of gene flow between *hammondii* and *atratus* either, but our sample from that specific area was rather small.

We have biochemical data on a sample of 42 specimens from Shasta and Tehama counties (Table 1) in north-central California, where Fox (1951) has mapped the range of *couchii* (*couchii* subgroup) as contacting that of *hydrophilus* (*atratus* subgroup). Twenty-four of these snakes were collected in north-central Shasta County (Fig. 2, open arrow), where the two subspecies have been presumed to intergrade (Fitch, 1940). Rossman and Stewart (personal communication) identified all of these specimens on morphological grounds as either *couchii* or *hydrophilus*. They did not find a single unquestionable intergrade, although they collected both taxa in the Pit River at the mouth of Deep Creek. Protein phenotypes of these snakes were in complete concordance with the identifications of Rossman and Stewart. One specimen of *couchii* (LSUMZ 9075) collected east of Shingletown in Battle Creek, a locality 25 miles away from the zone of contact between *couchii* and *hydrophilus*, did have a hybrid transferrin genotype (Trf<sup>d</sup>/Trf<sup>e</sup>), but its Sod phenotype was typical for *couchii*.

While such evidence is highly suggestive, contradictory findings by Stevan J. Arnold (personal communication) must be investigated before a definitive decision can be made on the species status of the two subgroups. Dr. Arnold has collected snakes that he identifies morphologically as hybrids of both *hammondii* X *atratus* and *hydrophilus* X *couchii*. Since he has also been able to successfully hybridize individuals of both pairs of taxa, in the laboratory, he is understandably reluctant to consider species status for these subgroups. A number of questions are suggested by these discordant findings: (a) Can hybrids between taxa of the two subgroups be identified unequivocally on morphological grounds? (b) If so, what morphological criteria should be used? (c) If natural hybrids do occur, with what frequency do they occur in the population? (d) Are individuals with protein phenotypes predicted for backcrosses also commonly present in the population? (e) Have sufficient specimens from areas of sympatry or parapatry been examined biochemically? It must be remembered that neither the successful hybridization of organisms under laboratory conditions nor the occurrence of rare natural hybrids is sufficient justification for deciding that populations are undergoing introgression in nature.

(c) Affinities of *Thamnophis ordinoides*

*Thamnophis ordinoides*, surprisingly, clustered with the *atratus* subgroup of *T. couchii* rather than with *T. elegans*. This finding is counter to the

opinions of previous students of the complex. Both Fitch (1940) and Fox (1948) commented on the morphological and ecological similarities of *T. ordinoides* and *T. e. terrestris*. Both snakes are terrestrial in habitat preference and feed primarily on slugs. Fox (1948) based his decision to give *T. ordinoides* species status on the careful examination of broods of gravid females of *T. ordinoides* and *T. e. terrestris* collected in northwestern California where the two forms are sympatric (Figs. 1 and 2). *T. ordinoides* also shows no sign of intergradation with the other subspecies of *T. elegans* (*vagrans* and *elegans*) whose ranges it overlaps (Fitch, 1940; Johnson, 1947).

The Nei distances separating *T. ordinoides* from the *atratus* subgroup of *T. couchii* are small, averaging 0.053 (0.017 to 0.091; Table 6). *T. c. hydrophilus*, which is the only subspecies of *T. couchii* sympatric with *T. ordinoides* (Fig. 2), is clearly separated from *T. ordinoides* on both ecological and morphological grounds (Fitch, 1940). The only allele unique to *T. ordinoides* was Trf<sup>e</sup> with a frequency of 0.177 (Table 3). Since at least 4 fixed alleles are involved, it is unlikely that this close molecular resemblance of *T. ordinoides* to the *atratus* subgroup is ascribable to undetected differences in allozyme mobilities.

The small Nei distance separating *T. ordinoides* from members of the *atratus* subgroup suggests that these forms have become reproductively isolated in relatively recent times. Probably speciation involved many changes at the behavioral and morphological levels where character divergence may occur more rapidly than at the protein level (King and Wilson, 1975). Speciation with little protein differentiation has also been described for *Thamnophis sauritus* and *T. proximus*, which are distinguished by a Nei distance of only 0.023 (calculated from the data of Gartside *et al.*, 1977); the phenomenon is also well documented for a variety of unrelated organisms, e. g. *Drosophila* (Carson *et al.*, 1975), minnows (Avisé *et al.*, 1975), and pocket gophers (Nevo *et al.*, 1974).

#### SUMMARY AND CONCLUSIONS

Previous immunological studies on transferrins indicate that all natricine snakes of North America are close relatives, having diverged from a common ancestor in the Pliocene (Mao and Dessauer, 1971). Species formation within the *elegans-couchii-ordinoides* complex undoubtedly has occurred much more recently. Microcomplement fixation comparisons show that structures of transferrins of members of the complex differ by only 5 to 15 immunological units (George, 1969; George and Dessauer, 1970; Dessauer,

unpubl. data), values indicative of divergence times within the past 1 to 3 million years.

The electrophoretic evidence suggests that the complex now consists of at least 3, and possibly as many as 4, species. (1) The most widely divergent species is *Thamnophis elegans*, which includes four very closely related subspecies: *T. e. elegans*, *T. e. terrestris*, *T. e. vagrans*, and *T. e. biscutatus*. The molecular evidence offers no argument against Rossman's (1979) decision to sink *T. e. biscutatus*; we recognize it herein solely as a matter of convenience in comparing data. (2) *Thamnophis couchii* includes two divergent groups of subspecies. *T. c. couchii* and *T. c. hammondii* (*couchii* subgroup) are separated from *T. c. atratus*, *T. c. aquaticus*, *T. c. gigas*, and *T. c. hydrophilus* (*atratus* subgroup) by a relatively large Nei genetic distance and fixed alleles at 2 loci. Contradictory findings must be investigated before a definitive decision can be made on whether or not the two subgroups represent two species. (3) *Thamnophis ordinoides*, which appears to be a good species, is remarkably close to the *atratus* subgroup, being distinguished from the latter by a genetic distance of similar magnitude to those differentiating subspecies within the *atratus* subgroup. Strangely, the morphology and ecological preferences of *T. ordinoides* are more similar to those of *T. elegans* than to *T. couchii*.

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