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GENETIC VARIATION AMONG POPULATIONS OF EASTERN NEWTS, *NOTOPHTHALMUS VIRIDESCENS:* A PRELIMINARY ANALYSIS BASED ON ALLOZYMES

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Abstract: The eastern newt, *Notophthalmus viridescens*, is widely distributed in eastern North America and has been divided into four subspecies. These subspecies differ geographically in morphology and life history, suggesting that the subspecies represent locally adapted and differentiated entities between which gene flow is significantly reduced. We investigated the relationships among subspecies by assessing population genetic structure across the range of the species. We analyzed 18 allozyme loci to examine the evolutionary relationships among the four subspecies of eastern newts: *N. v. viridescens*, *N. v. dorsalis*, *N. v. louisianensis*, and *N. v. piaropicola*. Despite moderate amounts of genetic variation, phylogenetic and phenetic analyses of the relationships among 12 sites resulted in trees that were inconsistent with the current subspecific classification. Cluster and phylogenetic analyses of allele frequency variation confirmed this, indicating an absence of significant differentiation among subspecies. Instead, populations of *N. viridescens* appear to cluster into groups representing geographic units that do not directly correspond to the currently recognized subspecies. The morphological and life history differences among the subspecies are not clearly associated with differentiation at allozyme loci. Recent divergence, gene flow, or phenotypic plasticity may explain the lack of correlation between genetic and morphological differentiation.

Key words: Allozymes; Gene flow; *Notophthalmus viridescens*; Salamandridae; Speciation

The Eastern newt, *Notophthalmus viridescens*, is a widely distributed species of salamanders in North America and is found from Nova Scotia to the Gulf of Mexico and from the eastern coast to southwestern Ontario and Texas (Fig. 1). This species has one of the most complex and variable life cycles of any North American salamander. Four life history stages are identified in many populations: (1) egg, (2) aquatic larva, (3) terrestrial "red eft" which, after 3–7 yr on land (Healy, 1974), transforms into (4) an aquatic adult (reviewed by Petranka, 1998). The duration and timing of the breeding season of *N. viridescens* varies according to latitude and climate. In northern populations, egg laying occurs in spring, whereas in southern populations egg laying may occur in early winter (reviewed by Petranka, 1998). The eft stage may represent a mechanism for reducing intraspecific competition (Healy, 1974) or for increasing dispersal (Gill, 1978). However, the eft stage is apparently lacking in some coastal populations (Harris, 1987). Four subspecies are recognized (Conant and Collins, 1998; Mecham, 1967): *N. v. viridescens*, *N. v. dorsalis*, *N. v. louisianensis*, and *N. v. piaropicola*. The parapatric distribution of these four subspecies suggests that morphological and life history variation within *N. viridescens* is geographically structured into locally adapted forms (Table 1).

Three previous studies have examined geographic variation in *N. viridescens*. Tabachnick (1977) used allozymes to differentiate nine populations of *N. v. viridescens* in New England based on five loci. He found that genetic variation correlated with seven environmental factors, but he did not find enough variation to delineate additional subspecies within *N. v. viridescens*. Merritt et al. (1984) also examined variation in 14 populations of *N. v. viridescens* in New England and found high levels of genetic similarity. Reilly (1990) used allozymes to examine the phylogenetic relationship of the three species of *Notophthalmus* and additionally examined seven populations of *N. viridescens* (three from *N. v. louisianensis*, two from *N. v. viridescens*, one from both *N. v. piaropicola* and *N. v. dorsalis*). He found relatively low levels of genetic variation within *N. viridescens*. None of the previous studies was specifically designed to examine genetic structure across the entire range of *N. viridescens*, including all four named subspecies.

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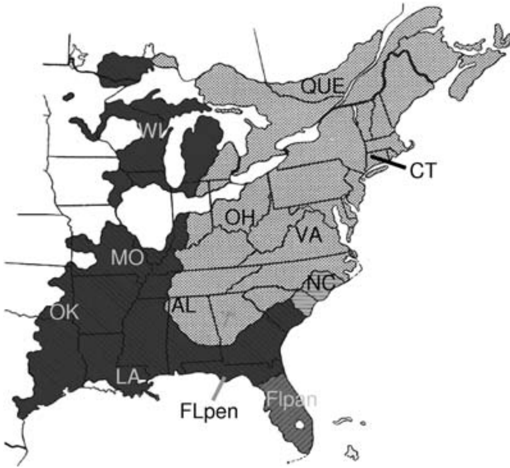

FIG. 1.—Localities sampled for the analysis of geographic variation in allozymes in *Notophthalmus viridescens*. (1) Alabama (AL), *N. v. v.*, (2) Connecticut (CT), *N. v. v.*, (3) Ohio (OH), *N. v. v.*, (4) Quebec (QUE), *N. v. v.*, (5) Virginia (VA), *N. v. v.*, (6) Florida peninsula (Flpen), *N. v. l.*, (7) Louisiana (LA), *N. v. l.*, (8) Missouri (MO), *N. v. l.*, (9) Oklahoma (OK), *N. v. l.*, (10) Wisconsin (WI), *N. v. l.*, (11) North Carolina (NC), *N. v. d.*, (12) Florida panhandle (Flpan), *N. v. p.* (Modified from Conant and Collins (1998). See Table 2 for locality and collection data for allozyme samples. *N. v. v.* = *N. v. viridescens*, *N. v. d.* = *N. v. dorsalis*, *N. v. l.* = *N. v. louisianensis*, and *N. v. p.* = *N. v. piaropicola*.

Notophthalmus v. viridescens, the red-spotted newt, is found in the Canadian Maritime Provinces to the Great Lakes and south to central Georgia and Alabama (Fig. 1). Members of this subspecies have red dorsal spots encircled by black (Petranka, 1998; Table 1). Breeding season varies according to the latitude and climate. In some populations, red-spotted newts migrate to the ponds and mate in fall through very early summer. While the mating season is extensive, females only lay eggs until spring (Gill, 1978; Healy, 1974; Hurlbert, 1969; reviewed by Petranka, 1998). In late summer, northern red-spotted newts return to terrestrial hibernacula as the ponds may freeze during winter. In more southern regions, adults do not always return to terrestrial sites. Aquatic larvae develop in the pond and generally transform into red efts at the end of summer. Most efts and adults are philopatric, but a few red efts disperse (Gill, 1978), which suggests that there is a possibility for gene flow between populations. Some populations, however, are presumably neotenic and, hence, lack the eft stage (Healy, 1974).

TABLE 1.—Summary of morphological differences between currently recognized subspecies of *Notophthalmus viridescens*.

Subspecies	Coloration[1]*	Size (cm)[2]†
N. v. viridescens	Dorsal spots encircled by black	5.7–12.2
N. v. louisianensis	Usually lacks red spotting on back	6.4–10
N. v. dorsalis	Broken red dorsolateral stripes that are bordered in black	6.4–9.5
N. v. piaropicola	Dark olive to black above, lacks red spotting, venter has black spots	7.5–10.5

[1]* Petranka (1998).
[2]† Conant and Collins (1998).

Notophthalmus v. louisianensis, the central newt, is found in central and southeastern North America, along the Gulf Coast and into Georgia and South Carolina (Fig. 1). Individuals are usually smaller than the red-spotted newt (C. R. Gabor, personal observation; Petranka, 1998) and usually lack red spots or the spots are small and not ringed with black (Table 1). Adults in southern populations breed in winter and spring (reviewed by Petranka, 1998). Individuals that live in swampy areas that stay wet throughout most of the year, such as in Louisiana and Florida, may never leave the aquatic habitat and may not have discrete breeding seasons (Goin, 1951; C. R. Gabor personal observation) and these populations also lack the eft stage.

Notophthalmus v. dorsalis, the broken-striped newt, is found in coastal North Carolina and South Carolina (Fig. 1). Individuals have broken, red, dorso-lateral stripes with black borders and are usually smaller than red spotted newts (Petranka, 1998; Table 1). They also lack the eft stage. Adults migrate to the pond, mate in fall, and leave the pond in early to mid-summer (Healy, 1974).

Notophthalmus v. piaropicola, the peninsula newt, is found in peninsular Florida (Fig. 1). It is usually smaller and more slender than the red-spotted newt (Petranka, 1998; Table 1). This newt is dark olive to dark brown above, lacks red spotting, and has a venter that is heavily marked with black spots. Little is known about these newts, but their environment is most similar to that of newts found in

TABLE 2.—Voucher specimens for tissues used for allozyme electrophoresis.

Species	Site name	LSUMZ Herpetology collection number	LSUMZ Tissue number	Locality	Latitude and longitude (decimal degrees)
Notophthalmus viridescens viridescens	Alabama	53491–500	H1220–29	Alabama: Lauderdale Co., Cox Creek	34.90N 87.64W
	Connecticut	53280–86	H987–93	Connecticut: Litchfield Co., Yale Forest	41.79N 73.24W
	Ohio	53514–23	H1165–74	Ohio: Scioto Co., Shawnee National Forest	38.80N 83.01W
	Quebec	55371–80	H1602–11	Quebec: Morgan Arboretum, Montreal	45.54N 74.11W
	Virginia	———	H2954–62	Virginia: Rockingham Co., George Washington National Forest	38.51N 78.88W
N. v. louisianensis	Florida (N) peninsula	54215–23	H1384–92	Florida peninsula: Bay Co., Pine Log State Forest	30.44N 85.88W
	Louisiana	48156–59	H38–41	Louisiana: Jefferson Parish, Jean Lafitte National Historic Park	29.80N 90.12W
	Missouri	53263–69	H980–86	Missouri: Crawford Co.	37.89N 91.30W
	Oklahoma	53251–57	H956–62	Oklahoma: Le Flore Co., Kiamichi Mountains	34.90N 94.70W
	Wisconsin	53342–50	H1033–41	Wisconsin: Portage Co.	44.47N 89.50W
N. v. dorsalis	North Carolina	———	H2963–66	North Carolina: Pender Co.	34.51N 77.89W
N. v. piaropicola	Florida (S) panhandle	53229–38	H882–91	Florida panhandle: Orange Co., St. Johns River	28.49N 81.29W

Louisiana and northern Florida (*N. v. louisianensis*).

The four subspecies of *N. viridescens* are defined by the morphological and life history characters described above; however, some authors have reported cases of apparent introgression between subspecies. Based on intermediate coloration and spotting patterns of species of *N. v. viridescens* and *N. v. louisianensis*, Ball (1998) concluded that all newts in Michigan are integrades between these two subspecies. Minton (1972) found a similar zone of introgression in Indiana. These findings suggest that gene flow between subspecies may be common.

We surveyed geographical patterns of variation in 18 allozyme loci to examine the evolutionary relationships among the four subspecies of *N. viridescens* (Fig. 1; Table 1). Phylogenetic, phenetic, and statistical analyses were used to test the hypothesis that the four nominal subspecies within *N. viridescens* represent locally adapted and differentiated taxa.

MATERIALS AND METHODS

Allozyme Methods

We used frozen tissue samples from the Louisiana State University Museum of Natural Science (LSUMZ) collection for starch gel allozyme electrophoresis. Liver and intestine were dissected from 96 individuals of both sexes from 12 sites (Fig. 1; Table 2) and stored at −76 C. Homogenates were subjected to horizontal starch gel electrophoresis following standard methods (Harris and Hopkinson, 1978; Selander et al., 1971). Twenty-one presumptive loci were surveyed, of which 18 were reliably scoreable and 17 were polymorphic (Appendix I). These 18 loci, listed under their respective buffer system used, along with abbreviations and IUBNC Enzyme Commission numbers, were as follows: *Poulik* (pH 8.7)—fumarate hydratase (FUM) (4.1.2.13), glycerol-3-phosphate dehydrogenase (GPD) (1.1.1.8), L-leucylglycyl-L-glycine substrate (LGG) (3.4.11). *Tris-Citrate II* (pH 8.0)—L-glutamate dehydrogenase (GLUD)

(1.4.1.2), L-lactate dehydrogenase (LDH, 2 loci) (1.1.1.27), Malate dehydrogenase (MDH, 2 loci) (1.1.1.37), L-phenylalanyl-L-proline substrate (PAP) (3.4.11). *Tris-Citrate II with NADP* (pH 8.0)—aconitate hydratase (ACON) (4.2.1.3), adenylate kinase (AK) (2.7.4.3), isocitrate dehydrogenase (ICD, 2 loci) (1.1.1.42), phosphoglucomutase (PGM) (5.4.2.2). *Tris-Citrate III* (pH 6.7)—Fructose biphosphate aldolase (ALD) (4.1.2.13), Esterase (EST) (3.1.1), glucose-6-phosphate isomerase (GPI) (5.3.1.9), 6-phosphogluconate dehydrogenase (PGD) (1.1.1.44). Mobility ratios were compared with those from previous runs, and questionable homologies were resolved by running samples in adjacent lanes. Electromorphs were lettered from anode to cathode.

Analyses

Allele frequencies were used to calculate pairwise genetic distances between all populations. The unbiased genetic distances (Nei, 1978) were calculated with BIOSYS-1 release 1.7 software (Swofford and Selander, 1981) and used to construct a UPGMA (Sneath and Sokal, 1973) dendrogram using PHYLIP (Felsenstein, 1995). However, because PHYLIP does not calculate Nei's (1978) unbiased genetic distances, bootstrap support was not estimated for this tree. PHYLIP was employed to calculate chord distances (Cavalli-Sforza and Edwards, 1967), which were analyzed using UPGMA and neighbor-joining methods (Saitou and Nei, 1987). Bootstrap support for these analyses was assessed from 1000 bootstrap replicates.

Trees were also constructed using minimum evolution and least square methods. Chord distances (Cavalli-Sforza and Edwards, 1967) and unbiased distances (Nei, 1978) were analyzed using the minimum evolution method of Rzhetsky and Nei (1992). A heuristic search using PAUP* version 4.0b10 (Swofford, 2002) was implemented with starting trees created from random stepwise addition (25 replicates), TBR branch-swapping and the MULTREES option. Bootstrap values for the minimum evolution trees were not obtained because PAUP* does not calculate genetic distances from allele frequency data. Chord distances (Cavalli-Sforza and Edwards, 1967) were also analyzed using the least squares method of Fitch and Margoliash (1967). The least squares analysis was performed using PHYLIP (Felsenstein, 1995) with global rearrangements of 10 random input orders of sites. Bootstrap values were determined from 1000 replicates.

Allele frequency data were also analyzed with continuous character maximum likelihood methods. This analysis was performed with the program CONTML of the PHYLIP software package (Felsenstein, 1995). Bootstrap support was assessed with 1000 replicates.

Nonmetric multidimensional scaling (MDS; Kruskal and Wish, 1978; Lessa, 1990) of Nei's (1978) genetic distances using the NCSS 2001 computer statistical package (NCSS Statistical Software, Kaysville, Utah) was used to illustrate the relationships among populations. This ordination technique allows for a visual inspection of the similarities among sites/OTUs and may be more appropriate than hierarchical (i.e., cluster and phylogenetic) analyses when reticulation or clinal patterns occur (Lessa, 1990).

Nested analysis of variance (ANOVA) was used to assess whether genetic variation was distributed along taxonomic (i.e., subspecies) lines following the methods of Weir and Cockerham (1984) and Weir (1996). Total sum of squares was partitioned into nested hierarchical components that represented variation among individuals within populations, among populations within nominal subspecies, and among subspecies using the ARLEQUIN version 2.0 software (Schneider et al., 2000). A second ANOVA was performed grouping populations into three regional groups (geographic regions) that were observed in the distance and MDS analyses. These geographic groups were: northern group (OH, WI, VA, CT, and QUE), southern group (OK, LA, MO, AL, and NC), and two Florida populations. The southern "group" is an artificial construction here. These southern populations are only loosely affiliated by geography; they do not form a coherent genetic cluster (See Results; Fig. 2). In this ANOVA, total sum of squares was partitioned into nested hierarchical components as above, with the highest level of the hierarchy representing the among-regional group variance component. These ANOVAs provide estimates of conventional, hierarchical F-statistics, called θ-statistics, using the formulas

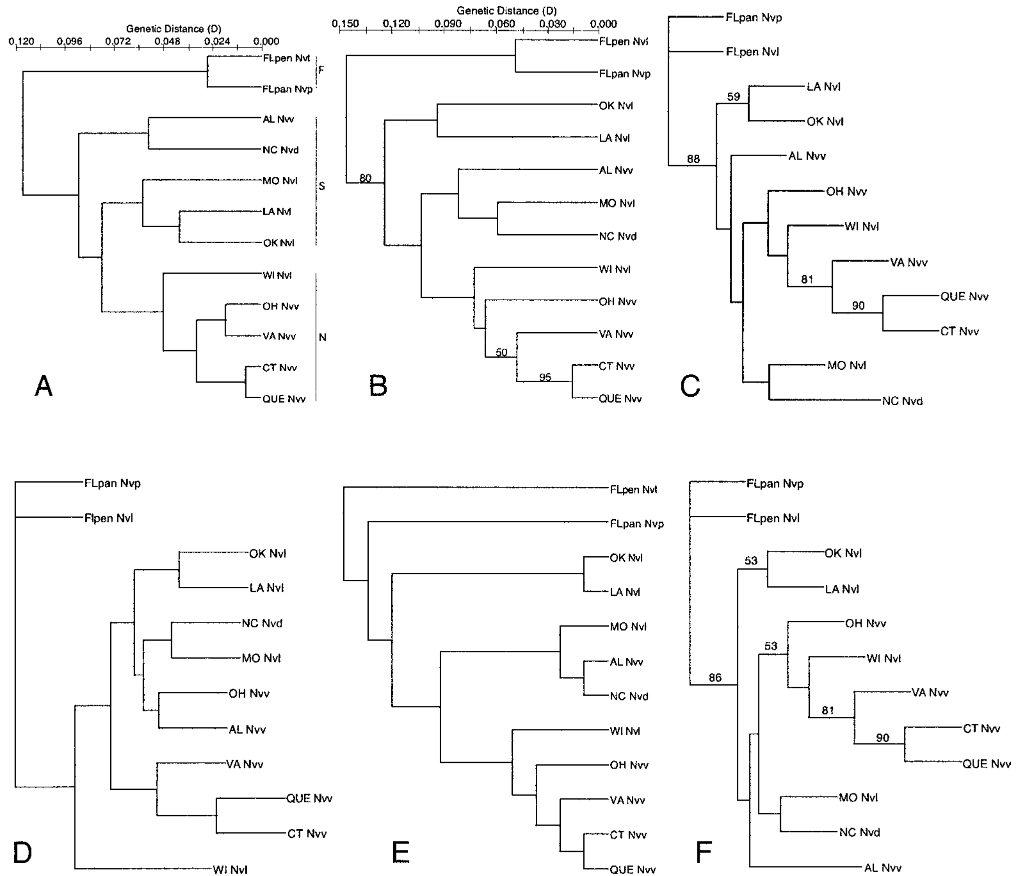

FIG. 2.—Dendogram illustrating relationships among the 12 sampled populations of *Notophthalmus viridescens*. (A) UPGMA dendogram using Nei's (1978) unbiased genetic distance. The cophenetic correlation is 0.876. No bootstrap values were obtained. Geographic regions are labeled to the right of the dendogram (N = north; S = south; F = Florida). (B) UPGMA dendogram based on Cavalli-Sforza and Edwards (1967) chord distance. (C) Neighbor-joining tree using Cavalli-Sforza and Edwards (1967) chord distances. (D) Maximum likelihood tree. (E) Minimum evolution tree based on Nei's (1978) unbiased genetic distances. No bootstrap values were obtained. (F) Least squares method tree based on Cavalli-Sforza and Edwards (1967) chord distances. Bootstrap values (as percentages) from 1000 replicates are above the branches. Site designations are the same as are used in Appendix I. *Nvv* = *N. v. viridescens*, *Nvd* = *N. v. dorsalis*, *Nvl* = *N. v. louisianensis*, and *Nvp* = *N. v. piaropicola*.

of Weir (1996) and Weir and Cockerham (1984). These statistics should not be confused with the Φ-statistics associated with molecular data. Significance of θ-statistics and variance components was determined by 1000 permutations of genotypes among sites and among subspecies under the null hypothesis of panmixia.

To provide a contrast to the ANOVA, tests for isolation-by-distance were also performed using the R-PACKAGE software (Legendre and Vaudor, 1991). We used Mantel tests (Mantel, 1967; Smouse et al., 1986) to determine the correlations between genetic distance (estimated as Nei's [1978] unbiased genetic distance) and geographic distance. Three isolation-by-distance analyses were performed. In the first, all sampled populations were included to test for overall patterns. In the second, the two Florida populations (the most distantly clustering populations [see Results]) were used as a basis for examining patterns in the rest of the populations. In the last analysis, correlations between genetic and

TABLE 3.—Results of hierarchical analysis (ANOVA) of allozyme variation in *Notophthalmus viridescens*. (A) Results with populations grouped by nominal subspecies, (B) Results with populations arranged into groups suggested by cluster analysis (see text; Figs. 2, 3).

Source of variation	df	SSD	Variance component	% of total	*P* value
A					
Among subspecies	3	8.4	0.020	4.35	0.17
Among populations/ Within subspecies	8	17.3	0.104	22.37	<0.001
Within populations	192	65.6	0.342	73.29	<0.001
Total	203	91.2	0.469		
B					
Among geographic regions	2	8.3	0.032	6.92	0.0156
Among populations/ Within groups	9	17.3	0.095	20.34	<0.001
Within populations	192	65.6	0.342	72.75	<0.001
Total	203	91.2	0.469		

geographic distances were examined within two groups defined by the clustering analyses described above (see Results). These two groups were comprised of northern populations (OH, WI, VA, CT, and QUE) and southern populations (OK, LA, MO, AL, and NC). Here again, we are treating the southern populations as a loose group, though they do not form a coherent genetic cluster. Separate tests of isolation-by-distance were performed to assess whether these population groupings showed similar patterns. A partial Mantel test was used to control for geographic distance between sites to examine the correlation between genetic distance and the subspecies and between genetic distance and the geographic regions.

RESULTS

Of the 18 allozyme loci scored in *N. viridescens*, only one locus, MDH-2, was monomorphic. Moderate amounts of genetic variation were detected but no fixed differences were observed between subspecies. The mean heterozygosity across all sites sampled was $\bar{H} \pm SD = 0.090 \pm 0.056$. Mean Nei's (1978) unbiased genetic distances between sites of *N. viridescens* was $\bar{D} \pm SD = 0.164 \pm 0.071$ (range D = 0.008–0.327).

Two major clusters of populations were observed in the UPGMA dendogram of genetic distances (Fig. 2A,B). One group consists of five northern populations: four of *N. v. viridescens* and one of *N. v. louisianensis*. A second cluster consists of the two sites sampled from Florida, each representing a different subspecies (Fig. 2A). The remaining five southern populations form a nebulous group with visible subgroups, one of which is more similar to the northern group than to the other southern group. This geographic clustering of populations is supported in most of the trees drawn using other distance metrics and methods. The northern group of populations appears as a monophyletic cluster in all trees except the maximum likelihood tree (Fig. 2D). The samples from Florida are closely related to each other (Nei's [1978] D = 0.049) and have high genetic distances to all other localities (range of D = 0.143–0.327; Table 4). The southern populations form two groups that are not distinctly differentiated from the northern group and the Florida populations in all trees except the maximum likelihood tree (Fig. 2). Despite differences in tree topologies, none of these analyses provide evidence of differentiation in accordance with the nominal taxonomic designations. The MDS analysis also revealed two clusters of populations. The remaining populations do not cluster together well and they vary in their positioning (Fig. 3). These groups are congruent with the geographic clusters of populations observed in the UPGMA dendograms and trees constructed by other methods described above (Fig. 2).

We used an analysis of variance (ANOVA) to partition genetic variation along subspecies boundaries and among geographic regions defined by the population trees and MDS analysis (Figs. 2, 3). There was no significant partitioning of genetic variation among subspecies ($\theta_{CT} = 0.043$, $P = 0.17$). The largest component of genetic variance was attributable to variation within populations (Table 3A). Grouping populations into geographic regions accounted for slightly more (6.9%) of the total variance ($\theta_{CT} = 0.069$, $P = 0.0156$), and this variance was significantly greater than

TABLE 4.—Nei's (1978) unbiased genetic distances (D) above the diagonal and geographic distance (km) after accounting for Earth's curvature below the diagonal for *Notophthalmus viridescens*, based on 18 loci scored in all sites. Abbreviations as in Appendix I.

Species		*N. v. viridescens*					*N. v. louisianensis*					*N. v. dorsalis*	*N. v. piaropicola*
		AL	CT	OH	QUE	VA	FLpen	LA	MO	OK	WI	NC	FLpan
N. v. viridescens	AL	—	0.186	0.130	0.219	0.161	0.236	0.139	0.142	0.193	0.216	0.088	0.262
	CT	1475	—	0.056	0.008	0.045	0.283	0.174	0.150	0.202	0.084	0.133	0.230
	OH	617	874	—	0.074	0.028	0.239	0.132	0.089	0.169	0.084	0.093	0.246
	QUE	1641	403	1026	—	0.056	0.327	0.196	0.184	0.229	0.121	0.169	0.292
	VA	848	641	311	895	—	0.258	0.148	0.127	0.184	0.077	0.115	0.236
N. v. louisianensis	FLpen	550	1734	1006	1993	1105	—	0.237	0.251	0.199	0.199	0.189	0.049
	LA	608	2003	1195	2218	1363	360	—	0.153	0.092	0.198	0.085	0.252
	MO	463	1581	737	1633	1043	991	930	—	0.192	0.181	0.072	0.253
	OK	643	2023	1153	2103	1438	967	738	477	—	0.200	0.131	0.186
	WI	1043	1350	826	1227	1082	1589	1607	698	1127	—	0.164	0.143
N. v. dorsalis	NC	874	940	640	1281	453	875	1206	1232	1516	1466	—	0.192
N. v. piaropicola	FLpan	967	1668	1178	2004	1131	526	859	1430	1485	1922	728	—

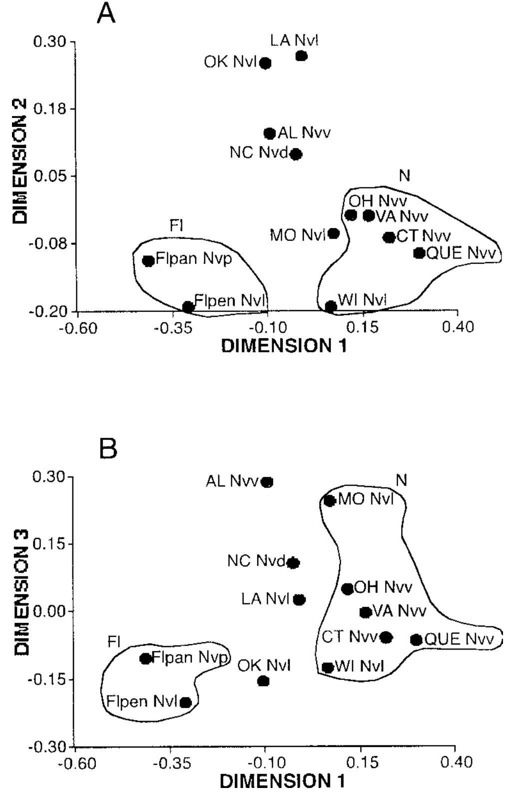

FIG. 3A,B.—Results of multidimensional scaling analyses, displayed as pairwise two dimensional scatterplots. (A) Dimension 1 versus dimension 2. (B) Dimension 1 versus dimension 3. The first two dimensions account for most of the variation and the first three dimensions account for virtually all of the variation. Localities and symbols for subspecies are labeled as in Fig. 2. Geographic regions are lettered as in Fig. 2A.

zero (Table 3B). In this second analysis of variance, the largest variance component was also attributable to variation within populations. The low θ_{CT} statistics indicate relatively low levels of genetic differentiation among geographic groups and insignificant differentiation along current taxonomic boundaries.

Nei's (1978) unbiased genetic distances and geographic distance (km, after accounting for Earth's curvature) between localities of *N. viridescens* are presented in Table 4. In the overall analysis of all populations, genetic distance was significantly positively correlated with geographic distance based on a Mantel test (Fig. 4A; $P = 0.005$, $r = 0.438$, 9999 replications). However, the intercept of the regression line was not zero (y-intercept = 0.091),

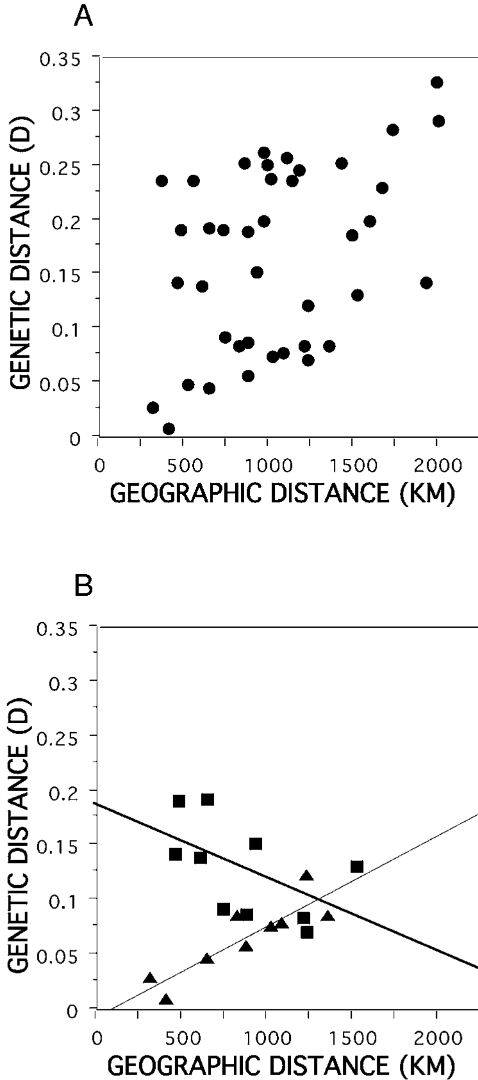

FIG. 4.—Nei's (1978) unbiased genetic distance (*D*) plotted against geographic distance (km) (after accounting for Earth's curvature) for: (A) all 12 sites of *Notophthalmus viridescens* at 18 loci, (B) the northern and southern sites against themselves (squares = south versus south, triangles = north versus north, thick line = the regression line for the comparison within the southern groups, thin line = the regression line for the comparison within the northern groups).

indicating that isolation-by-distance alone does not entirely explain the patterns of population genetic relationships.

This pattern is evident when the analysis is restricted to distances from the two Florida populations to all others. In these analyses, the y-intercepts are not close to zero (Fl pan y-intercept = 0.19; Fl pen y-intercept = 0.23). Inspection of pairwise genetic distances (Table 4) indicates, for example, that genetic distances between the Alabama population sample and the two Florida populations are substantially larger than expected when the proximity of these populations to each other is considered. There is a significant relationship between geographic distance and genetic distance in the northern sites (Fig. 4B; P = 0.019, r = 0.866, 9999 replications), with a y-intercept (–0.007) close to zero. There is not, however, a significant relationship between geographic and genetic distances among the southern sites (Fig. 4B; P = 0.035, r = –0.542, 9999 replications), with a y-intercept (0.187) not close to zero.

When we controlled for geographic distance between sites in a partial Mantel test, we found that the current subspecies status is not significantly correlated with genetic distance (P = 0.302, r = 0.072, 9999 replications). On the other hand, there was a significant correlation when the populations were artificially organized into three groups (northern, Florida, and an incoherent southern group, Fig. 2A) versus genetic distance with geographic distance controlled using a partial Mantel test (P = 0.0003, r = 0.618, 9999 replications). When the populations were organized by geography, the correlation with genetic distance better explained the patterns of genetic differentiation than did the current subspecies taxonomy (Figs. 2, 3).

DISCUSSION

Our survey of allozyme variation in *N. viridescens* found moderate levels of genetic differentiation; however, this variation was not organized along taxonomic (subspecies) lines. The mean heterozygosity across all populations of *N. viridescens* ($\bar{H} \pm SD = 0.090 \pm 0.056$) was lower than values reported by Reilly (1990: $\bar{H} \pm SD = 0.128 \pm 0.145$) and Merritt et al. (1984: $\bar{H} \pm SD = 0.153 \pm 0.02$) for *N. viridescens*, but the value reported here falls near the mid-point of values found among 102 species of salamanders surveyed by Shaffer and Breden (1989). Mean Nei's (1978) unbiased genetic distances between sites of *N. viridescens* ($\bar{D} \pm SD = 0.164 \pm 0.071$; range D = 0.008–0.327) was greater

than those found by Reilly (1990) and less than among species of *Taricha* (Hedgecock and Ayala, 1974). In Reilly's (1990) investigation, the maximum genetic distance was found between *N. v. piaropicola* and an Illinois population of *N. v. louisianensis* (D = 0.33). Mean Nei's distance among populations of *N. v. viridescens* from Reilly (1990) was $\bar{D}$ = 0.128. Tilley et al. (1990) found genetic distances ranging from 0.08 to 0.46 among sites of *Desmognathus ochrophaeus*, a wide-ranging plethodontid species from eastern North America. Tilley and Mahoney (1996) also found similar distances among five sites of *D. ochrophaeus*. They proposed splitting this species into four species from northeast to southwest because the distances between the sites (range of D = 0.223–0.528) was as much or more than between sympatric species of *Desmognathus* that do not hybridize (range of D = 0.235–0.564). These distrances are larger than what we observe for the four subspecies/OTUs examined. Eastern newts had higher pairwise genetic distances (Table 4) than the genetic distances reported between populations of another species of salamander with a large geographic range, *Ambystoma tigrinum*, from Georgia and Wisconsin ($\bar{D}$ = 0.117; Shaffer et al., 1991). On the other hand, eastern newts have much lower genetic distances compared to the genetic distance between *A. tigrinum* populations from California and Mexico ($\bar{D}$ = 0.59; Shaffer, 1984).

Despite moderate amounts of genetic variability, the current taxonomy within *N. viridescens* is not supported by the allozyme data presented here despite documented morphological and life history differences among subspecies. The lack of support for the current taxonomy is evident in the clustering and phylogenetic analyses (Figs. 2, 3); none of these analyses grouped population samples in accordance with their subspecific designation. The lack of support for the current taxonomy is confirmed by the analysis of allozyme allele frequency variance (Table 3A) in which there was no significant variance component attributable to differences among subspecies. Comparison of geographic and genetic distances suggests an alternative structure in the data: a pattern of isolation-by-distance (Fig. 4A). Indeed, much of the isolation-by-distance occurs along a north–south axis as is evident in dimension 1 of the MDS plot (Fig. 3). However, while there is a positive, significant association between geographic and genetic distance, the y-intercept for the regression line is not zero. This significant correlation between geographic and genetic distance indicates that isolation-by-distance is not an entirely adequate description of the distribution of population genetic variation within *N. viridescens*. Based on the MDS, trees, and examination of geographic variation and genetic distances, two biogeographical groups are identifiable that may represent distinct evolutionary units and the remaining populations do not form a coherent biogeographic unit. The Florida populations represent one of these units. The two populations sampled from Florida are relatively closely related to each other, but appear as a distinct group in all of the clustering and phylogenetic analyses (Figs. 2, 3). The other group is represented by a northern cluster (Fig. 2).

Populations in the northern and southern parts of the range of *N. viridescens* differ in the organization of their genetic variation. Among the northern populations, there is a significant, positive correlation between geographic and genetic distance (Fig. 4B) with the y-intercept near zero, indicating a pattern of restricted gene flow with isolation-by-distance. This pattern is distinctly different from the pattern observed in the southern populations where there was not a significant correlation between geographic and genetic distance (Fig. 4B). The pattern observed among the southern populations can be created by unrestricted gene flow or a complete cessation of gene flow. The southern group of populations appears to be isolated from each other and undergoing drift. Genetic distances among the southern populations are relatively high compared to the northern group (Fig. 4B; Table 4), suggesting that gene flow is limited in the south. A possible explanation for these alternative population genetic patterns involves differences in the Pleistocene-aged histories of the northern and southern portions of the range of *N. viridescens*. The northern portion of the range was uninhabitable during glacial maxima and *N. viridescens* must have recolonized this region after the retreat of the Laurentide ice sheets (Pielou, 1991). The populations in the southern portion of the range may therefore be

significantly older than the northern populations and this difference in relative age may explain the contrasting geographic-genetic patterns. The older southern populations have presumably had more time for differentiation leading to higher pairwise genetic distances among populations. The more recent colonization of formerly glaciated northern areas means that northern populations should be relatively less differentiated. Indeed, the lower pairwise genetic distances and the pattern of isolation-by-distance detected among northern populations supports this interpretation. Similar patterns have been observed in other salamanders, including *Plethedon cinereus* (Highton, 1999) and *Ambystoma tigrinum* (Templeton et al., 1995), as well as other organisms (e.g., Starkey et al., 2003).

Two hypotheses may explain the lack of congruence between the allozyme data reported here and the morphological and life-history variation that distinguishes the subspecies: (1) the allozyme variation observed within *N. viridescens* is representative of ancestral polymorphism and/or (2) selection maintains morphological and life-history variation among subspecies despite substantial gene flow that is occurring (or has occurred recently) between populations and subspecies. Taken together, the geographic distributions of genetic and morphological/life-history variation among subspecies suggest that morphological evolution has outpaced evolution at allozyme loci in *N. viridescens*. If the subspecies represent meaningful evolutionary units and subspecific morphology and life-history differences are heritable (i.e., not plastic responses to varying environments), they must have evolved relatively recently. If this is the case, the lack of differentiation among subspecies is due largely to insufficient time since divergence for lineage sorting to occur at the allozyme level. In other words, the variation observed from our allozyme survey likely represents ancestral polymorphism. Neigel and Avise (1986) pointed out that there is a potentially lengthy period after the onset of divergence during which variation at neutral markers may not converge to the boundaries of the diverging evolutionary units (the subspecies in this case).

The alternative to the hypothesis of ancestral polymorphism is one of strong selection that maintains morphological and life-history differences among subspecies despite substantial gene flow. The implication from Mantel tests is that while panmixia may be excluded, there is evidence of local gene flow with isolation-by-distance. Assuming an island model and equilibrium, the average pairwise F_{st} among populations of *N. viridescens* (F_{st} = 0.248) indicates that gene flow may be occurring at a rate of approximately Nm = 0.75 migrants per generation (Slatkin, 1987). Further evidence for this inference comes from the observation that most of the genetic variation is contained within populations, rather than between them (Table 3). If the morphological and life-history differences observed among subspecies are adaptive, selection may maintain linkage disequilibrium between ecologically relevant genes and neutral markers, allowing divergence despite gene flow. In fact, this disequilibrium between quantitative trait loci and neutral markers can be considered evidence of divergent selection (Whitlock, 1999).

Assuming that the allozyme variation reported here is neutral genetic variation, both hypotheses suggest that divergence within *N. viridescens* may be recent or ongoing. The hypothesis of ancestral polymorphism for neutral markers implies recency by definition. The alternative hypothesis of strong selection in the face of gene flow suggests that no effective barrier to reproduction between subspecies exists. If the subspecies represent locally adapted forms, we might reasonably expect such barriers to gene flow to evolve shortly after divergence. Evidence of possible gene flow between differentiated subspecies, therefore, also suggests that divergence within *N. viridescens* is a recent phenomenon. Distinguishing between these hypotheses will be difficult without further investigations including assessment of reproductive isolation at contact zones, greater geographic sampling, the employment of markers with greater resolution, as well as field studies of migration patterns. Further investigation of local selection regimes and the possibility of phenotypic plasticity may help explain the lack of significant genetic differentiation between populations that are different in life-history strategies (e.g., eft versus no eft stage). More detailed quantification of morphological variation may

also provide insight into the history of diversification within *N. viridescens*.

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APPENDIX I

The distribution of alleles among the 12 sites for 18 loci sampled in the analysis of geographic variation in *Notophthalmus viridescens*. Letters designate alleles. N = sample size, AL = Alabama, CT = Connecticut, OH = Ohio, QUE = Quebec, VA = Virginia, FLpen = Florida peninsula, LA = Louisiana, MO = Missouri, OK = Oklahoma, WI = Wisconsin, NC = North Carolina, FLpan = Florida panhandle. *H* = mean heterozygosity, A/L = mean number of alleles per locus, and %P = percent loci polymorphic.

	Localities											
Subspecies	*N. v. viridescens*					*N. v. louisianensis*					*N. v. dorsalis*	*N. v. piaropicola*
Locus	AL	CT	OH	QUE	VA	FLpen	LA	MO	OK	WI	NC	FLpan
ACON												
(N)	9	7	7	6	4	10	4	7	7	9	1	9
A	0.11	0.21	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00
B	0.00	0.79	0.93	1.00	1.00	0.00	0.00	0.29	0.00	0.94	0.00	0.00
C	0.89	0.00	0.07	0.00	0.00	1.00	0.88	0.71	1.00	0.06	1.00	1.00
AK												
(N)	10	6	7	10	5	10	3	7	6	8	2	9
A	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00
B	1.00	1.00	0.93	0.95	0.70	1.00	0.67	1.00	0.92	1.00	1.00	1.00
C	0.00	0.00	0.07	0.05	0.30	0.00	0.00	0.00	0.08	0.00	0.00	0.00
ALD												
(N)	10	10	10	10	10	10	4	7	7	9	2	9
A	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.36	0.50	0.00	1.00
B	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	0.64	0.50	1.00	0.00
EST												
(N)	10	7	10	10	10	10	4	5	6	9	4	8
A	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B	0.00	0.36	0.05	0.65	0.50	0.00	0.38	0.00	0.00	0.00	0.13	0.00
C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00
D	0.00	0.64	0.00	0.30	0.20	0.10	0.00	0.10	0.00	0.83	0.13	0.94

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The distribution of alleles among the 12 sites for 18 loci sampled in the analysis of geographic variation in *Notophthalmus viridescens*. Letters designate alleles. N = sample size, AL = Alabama, CT = Connecticut, OH = Ohio, QUE = Quebec, VA = Virginia, FLpen = Florida peninsula, LA = Louisiana, MO = Missouri, OK = Oklahoma, WI = Wisconsin, NC = North Carolina, FLpan = Florida panhandle. *H* = mean heterozygosity, A/L = mean number of alleles per locus, and %P = percent loci polymorphic.

Continued.

	Localities											
Subspecies	*N. v. viridescens*					*N. v. louisianensis*					*N. v. dorsalis*	*N. v. piaropicola*
Locus	AL	CT	OH	QUE	VA	FLpen	LA	MO	OK	WI	NC	FLpan
E	0.00	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00	0.00	0.00
F	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.92	0.00	0.00	0.00
G	1.00	0.00	0.40	0.00	0.00	0.55	0.63	0.60	0.00	0.17	0.75	0.06
H	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
I	0.00	0.00	0.00	0.00	0.30	0.00	0.00	0.30	0.00	0.00	0.00	0.00
J	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
K	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FUM												
(N)	10	6	10	5	10	10	4	7	7	9	1	9
A	0.00	0.67	0.00	0.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B	1.00	0.33	0.95	0.20	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLUD												
(N)	6	6	10	8	7	9	2	3	5	1	1	3
A	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
B	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00
GPD												
(N)	10	10	10	10	10	10	4	7	7	9	1	9
A	1.00	1.00	0.70	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00
C	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GPI												
(N)	10	10	10	10	10	10	4	7	6	9	1	9
A	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.06	0.00	0.11
B	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	0.89	1.00	0.89
C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00
ICD-1												
(N)	9	7	10	10	9	10	4	7	7	9	1	9
A	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B	0.11	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C	0.89	1.00	0.80	1.00	1.00	1.00	0.88	1.00	1.00	1.00	1.00	1.00
D	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00
ICD-2												
(N)	10	7	10	10	10	10	3	7	7	9	1	9
A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.00	0.00
B	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	1.00	0.44	1.00	1.00
C	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
LDH-1												
(N)	9	7	10	9	9	10	4	7	7	7	4	8
A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06
B	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.94
C	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.43	0.00	0.00	0.00
D	0.00	0.00	0.00	0.06	0.06	0.00	0.00	0.00	0.00	0.50	0.00	0.00
E	1.00	1.00	1.00	0.94	0.94	0.00	0.88	1.00	0.57	0.50	1.00	0.00

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Continued.

	Localities											
Subspecies	*N. v. viridescens*					*N. v. louisianensis*					*N. v. dorsalis*	*N. v. piaropicola*
Locus	AL	CT	OH	QUE	VA	FLpen	LA	MO	OK	WI	NC	FLpan
LDH-2												
(N)	9	7	10	10	10	10	4	7	7	9	4	9
A	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.11
B	0.00	0.00	0.00	0.00	0.00	0.10	0.25	0.00	0.00	0.00	0.00	0.00
C	1.00	1.00	1.00	1.00	0.95	0.85	0.75	1.00	1.00	1.00	0.88	0.78
D	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.13	0.00
E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11
LGG												
(N)	10	7	10	10	10	10	4	7	7	9	4	9
A	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B	0.00	1.00	1.00	0.90	0.90	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C	1.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MDH-1												
(N)	10	7	10	10	10	10	4	7	1	9	4	10
A	0.00	0.14	0.00	0.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
C	1.00	0.86	1.00	0.65	1.00	1.00	0.88	0.86	1.00	1.00	1.00	0.90
D	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.07	0.00	0.00	0.00	0.00
E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00
MDH-2												
(N)	10	7	10	10	10	10	4	7	7	9	4	9
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PAP												
(N)	10	10	10	10	10	9	4	7	7	9	4	9
A	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B	0.00	0.00	0.00	0.00	0.00	0.00	0.88	0.00	0.43	0.00	0.00	0.00
C	1.00	1.00	0.95	0.85	1.00	1.00	0.13	1.00	0.00	1.00	0.88	1.00
D	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00
E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.57	0.00	0.00	0.00
PGD												
(N)	10	7	10	10	10	10	4	7	7	9	4	9
A	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00
B	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.00
C	0.00	0.00	0.00	0.00	0.20	0.00	0.13	0.00	0.00	0.06	0.00	0.28
D	1.00	1.00	0.95	1.00	0.80	0.30	0.88	1.00	1.00	0.94	1.00	0.72
E	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PGM												
(N)	9	7	10	9	10	10	4	8	6	9	3	9
A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.67	0.06
B	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C	1.00	1.00	1.00	1.00	0.85	1.00	1.00	0.69	1.00	1.00	0.33	0.94
H	0.018	0.05	0.083	0.073	0.095	0.09	0.245	0.067	0.093	0.129	0.079	0.062
A/L	1.2	1.2	1.7	1.4	1.4	1.4	1.6	1.3	1.3	1.4	1.3	1.4
%P	16.7	22.2	44.4	38.9	38.9	22.2	55.5	22.2	33.3	38.9	22.2	38.9